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PRINCIPAL INVESTIGATOR:
Serge Przedborski, M.D., Ph.D

CONTRACTING ORGANIZATION:
Columbia University
New York, NY 10032

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease for which there is no cure. Because of the lack of understanding of the cause of ALS, treatment is limited to mechanical intervention and riluzole, both of which extend existence rather than extend life. Thus, there is an urgent need to find drugs that are of some benefit to ALS. To this end, we are developing a cell-based co-culture high throughput screening system which is composed of mutant SOD1 astrocyte-conditioned medium (ACM), ES-MNs and a high-throughput screening robot, that we believe, will give faster screening of chemicals and drugs to treat ALS. We have, thus far, validated our co-culture system, validated that our high throughput screening technique works and miniaturized our system to a 96 well plate. Z'-factor (between 0.5-1.0) is a measure of how good an assay is. At present, our Z'-factor is 0.3, and we are working on improving this value. Nonetheless, we have identified 108 compounds, from our first screen from chemical libraries, that have a wide range of actions. Of these 108 compounds, five have reached the second screen stage and two of the five hold good possibilities. With our system, we noted that JNK2/3 inhibitors are protective for primary motor neurons and ES-MNs) which was confirmed on a low throughput screen. Compared to other high throughput screening systems, our readout is simple: survival of ES-MNs in ACM treated with one of the hits from our high throughput screen coculture system seems to be successful in finding small molecules that are possibilities for the treatment of ALS.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal paralytic disorder of the voluntary motor system (1). The onset of this disease usually occurs between the fourth and fifth decade of life and both age and a positive family history are absolute risk factors (2, 3). Noted recently is the fact that US military serving in the Persian Gulf War show an increased incidence of ALS (2). Clinically, ALS is characterized by muscle weakness, spontaneous muscle contractions, and extensor plantar responses. Although motor deficits usually predominate in the limbs, bulbar innervation can be severely involved early in the course of the disease, leading to atrophy of the tongue, dysphagia, and dysarthria (4). Disease progression is rapid, and once diagnosed, depending on the mutation, survival time is about three years (5). Pathologically, ALS is characterized mainly by a loss of the upper motor neurons (MNs) or the lower MNs or both (4). To date, there are only a few approved treatments for ALS, such as mechanical intervention (usually a respirator) and riluzole (little is known about this drug which has glutaminergic activity), that do prolong survival to a limited extent (6). Because we have so little knowledge of the mechanisms by which MNs die in ALS, the development of any effective suppressive or neuroprotective therapies has been quite limited.

ALS is essentially a sporadic condition, however, about 10% of the disease is inherited (4). Most familial forms of ALS are autosomal dominant and are clinically and pathologically almost indistinguishable from sporadic ALS. However, familial ALS has an earlier age of onset, a more rapid course of development and progression, and a survival after diagnosis of only 1.5 years (5). About 20% of familial ALS cases are linked to mutations in the gene encoding the enzyme SOD1 (5). To date, more than 140 mutations in this enzyme have been identified in familial ALS families (5) and regardless of the mutation in SOD, the outcome is the same. Because of the similarity between the sporadic and the familial forms of the disease, particularly in terms of pathogenic mechanisms (6), information gleaned from studying ALS caused by mutant SOD1 may possibly reveal some of the key cellular and molecular mechanisms of sporadic ALS, as well as possibly identify new therapeutic strategies for the treatment of this debilitating disease.

What do we know about SOD1? SOD1 is an abundant, ubiquitously expressed cytosolic enzyme whose only known function is to dismutate superoxide to hydrogen peroxide (7). Although SOD1 is thought of as essential for living organisms (7), mutant mice lacking this enzyme thrive normally and do not develop ALS (8). Conversely, transgenic (Tg) rodents expressing either catalytically active SOD1 mutants (9, 10) or catalytically inactive SOD1 mutants (11, 12) mimic both the clinical and the neuropathological hallmarks of ALS. Tg mice expressing high levels of wild-type human SOD1 are healthy (10) however, as these mice age, they develop considerable gait problems (personal observation) which, at some point, needs to be looked into. However, taken together, these findings argue for mutant SOD1 causing MN degeneration via a gain-of-toxic function. Despite intense research efforts, the nature of this adverse property of mutant SOD1 remains elusive. Proposals as to mutant SOD1 cytotoxicity involve several different mechanisms including oxidative stress (13, 14) protein aggregation (15) aberrant protein-protein interactions (16), decreased binding affinity for zinc (17)

mitochondrial dysfunction (18), excitotoxicity (19), glutamate transporter failure (20), endoplasmic reticulum stress (21) and apoptosis (22, 23), none of which are mutually exclusive.

Several studies have shown that both the selective lowering of mutant SOD1 in either MNs or in glial cells such as microglia and, more recently, astrocytes, by a Cre-Lox system prolongs survival in Tg SOD1^{G37R} mice compared to their germline littermates (24, 25). Using a neuronal/glial co-culture system, we and others (26-29) have found that astrocytes expressing mutant SOD1 trigger the death of primary and embryonic stem (ES)-cell derived MNs. We further showed that the contribution of the mutant astrocytes to the death of MN *in vitro* is caused by a soluble factor (26) which seems to be manufactured by the mutant astrocytes. Indeed, media conditioned by mutant SOD1 astrocytes impair MN survival while media conditioned by mutant microglia do not (26). Furthermore, MNs are selectively vulnerable to this mutant SOD1 astrocyte-derived toxic factor, whereas GABA interneurons and dorsal root ganglion neurons are not (26). Mutant SOD1 astrocytes or medium conditioned with mutant SOD1 astrocytes represent part of the backbone of our high throughput screen system to screen compounds that just might be useful for the treatment of ALS.

Several *in vitro* models of ALS do exist, however, compared to these currently available *in vitro* ALS models, our recently developed co-culture system (27), composed of mutant SOD1 astrocytes and embryonic stem cell-derived MNs (ES-MNs), has four essential advantages over other *in vitro* models: (i) ES-MNs combine both expandability and characteristics of mature MNs; (ii) our model system shows a spontaneous neurodegeneration directly linked to an animal model of the disease that emulates a known human condition; (iii) mutant astrocyte-mediated toxicity is specific to MNs; and, (iv) this culture system can model processes both extrinsic and intrinsic to MNs, which is vital given the demonstrated cell-autonomous and non-cell-autonomous components of familial ALS. Thus, we think that we have a unique tool which we can use to identify small molecules that may be neurosuppressive or neuroprotective in ALS. This system increases the possibility of getting ALS medications to the patient much earlier. In this respect, we have put forth experiments to develop and refine our high throughput screen system as well as to identify molecules that may be useful for the treatment of ALS.

Body of Research

The goal of this project is to identify small molecules that could be useful for the treatment of ALS. As mentioned above, the only two treatments that have been approved for this debilitating disease are mechanical intervention and riluzole. These are of limited value in that they do not slow or stop the progression of the disease and are used primarily when the patient is close to endstage to really extend existence' but not life. What is needed here are compound(s) that will stop or slow the progression of ALS, but to do this, we must not only unravel the mechanisms involved in the initiation and production of this debilitating disease, at the same time, we must screen molecules to find potential candidates for further study. Screening molecules can be a slow and

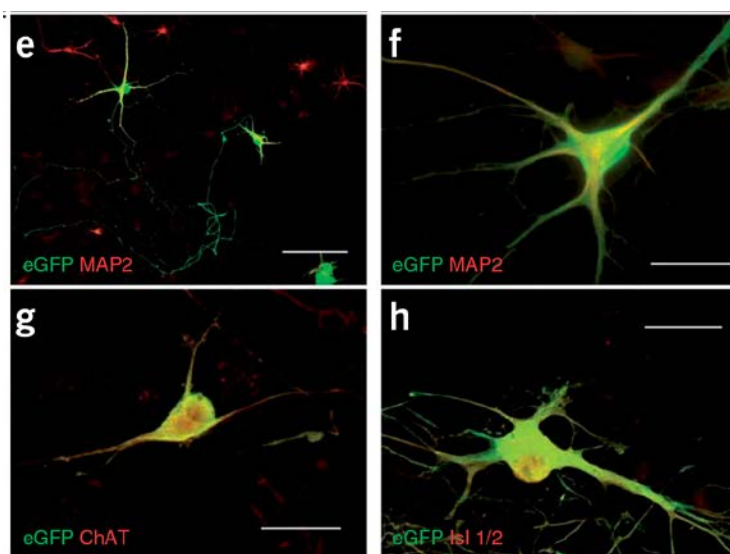
tedious process and can involve an enormous amount of time and effort. Since we have found that mutant SOD1 astrocytes produce a factor(s) that is toxic to spinal motor neurons (27) and to stem cell-derived MN (ES-MN) (30), we surmised that, rather than trying to culture spinal MNs from spinal cords of mutant SOD1 mice, we could use these ES-MNs instead and they work just as well. Therefore, using the toxic effect of mutant SOD1 astrocytes and the availability of ES-MNs in a co-culture system would allow us to screen several small molecule libraries in record time. For this, we put forth a series of Specific Aims (SA) to develop a high throughput screening method to screen small molecule libraries in search for compounds that might be useful for the treatment of ALS. In **SA I**, we planned to perform a primary high-throughput screen using our *in vitro* culture system to screen two small chemical libraries, the Columbia Library and the Harvard Library for neuroprotective molecules of direct relevance to ALS. These libraries consist of 80,000 compounds and 100,000 compounds, respectively. At this point, the aim was to identify 100 possible compounds. For **SA II**, we planned to put the 100 identified small molecules, found in SA I. through a low throughput screen. These 100 compounds were to be tested for their ability to protect human MNs derived from Presidential human ES cell lines. This second aim is performed to narrow the list of possible compounds to about 20. **SA III** was to consist of toxicity and pharmacokinetic studies on the 20 noted compounds. All of the compounds that are found to be suitable would be tested in parallel at Columbia and Harvard Universities for cross-validation. It was expected that through these three specific aims, we would identify 2-3 compounds for further testing.

Key Accomplishments

Year 1 (2008-2009)

ALS is a neurodegenerative disease in which MNs are the primary affected cell population. From Dr. Chris Henderson, a C0-Director of the Motor Neuron Center here

Figure 1. Mouse ES-MNs



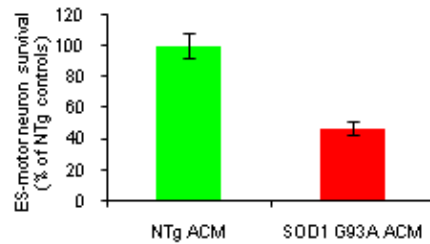
Scale bars, 50 μ m (f–h) and 100 μ m (e).

(from Nagai et al., Nat. Neurosci.2007)

at Columbia, we learned how to culture primary MNs. While this type of *in vitro* preparation is part of the backbone of our future secondary screening, it is impossible to generate large numbers of primary MNs from Tg mutant SOD1 animals with sufficient regularity to perform the high-throughput screen of the type planned here. Thus, we have learned the experimental conditions required for the *in vitro* differentiation of ES cells into MNs (30) which provides us with an infinitely expandable

source of cells shown in Figure express enhanced protein (GFP) specific promoter immunopositive for protein-2 (MAP2; transferase 1/2 (h). These shown to develop receptors and necessary for action potential firing and functional synapses with muscle fibers (31).

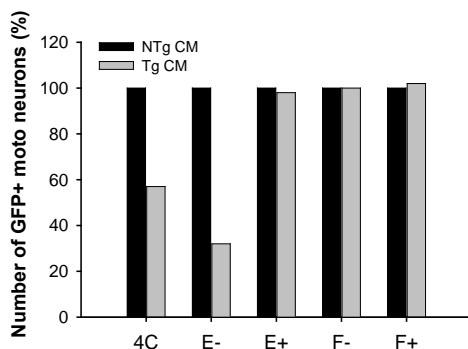
Figure 2 FACS-sorted ES-MNs survival in NTg and mutant astrocyte conditioned medium.



relevant to ALS. As 1, these mouse ES-MNs green fluorescent under control of the MN-*HB9* and are microtubule associated e,f), choline acetyl (ChAT; g) and Islet (Isl) cells have also been appropriate transmitter intrinsic properties appropriate patterns of

As the primary source of cells for this project, we need highly purified MNs. So far, we have tested two techniques for the purification of these cells: 1) fluorescence activated cell sorting or FACS, based on the expression of green fluorescent protein (GFP) by both our ES-derived and primary motor neurons produced from Tg *Hb9::GFP* mice; and, 2) immunoaffinity magnetic column sorting or MACS, based on the transgenic expression of the antigen CD2 by our HB9-GFP ES-MNs. By FACS, we have obtained 100% purity in GFP⁺ MNs, and as illustrated in Fig. 2, ES-MNs remain vulnerable to the toxicity of mutant SOD1 astrocytes. For the sorting by MACS, we have obtained about

Figure 3. Compared to control mutant conditioned medium kept at 4°C (4C), the toxic activity is recovered only in the eluate of the anionic column (E-).



F = flow through; E+ = eluate from cationic column.

85-95% purity in MNs, and a comparable vulnerability (not shown) to mutant astrocytes, using the CD2 genetically-engineered ES-MNs. Thus, at this point we already have in hand one effective method, i.e., FACS, to achieve an optimal purification of ES-MNs (Figure 2).

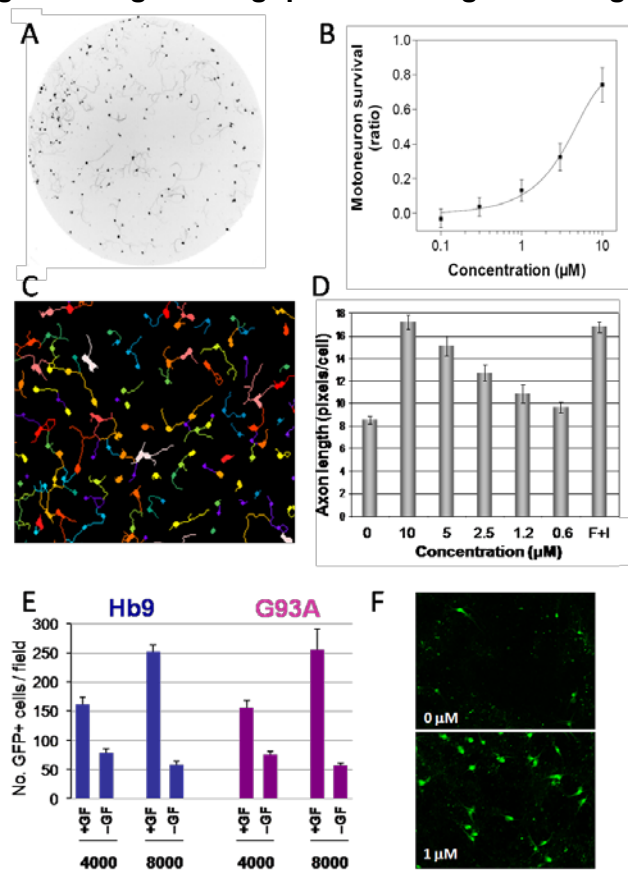
We have found that the toxic activity produced by mutant SOD1 astrocytes can be captured on a Q Sepharose™ XL strong anion exchange but not on a SP Sepharose™ XL strong cation exchange column (Figure 3). More importantly, on such chromatographic purification, the toxicity of the mutant conditioned medium is augmented in that before purification, it kills on average 50% of the ES-MNs whereas after purification, it kills on average 70% of the ES-MNs (Figure 3). Note that after similar purification, wild-type conditioned medium remains non-toxic to ES-

MNs. We also demonstrated that the toxic activity of the mutant conditioned medium is stable at 4°C for at least 4-5 days and is completely lost after protease treatment (pepsin-A, 0.2 mg/mL, 2 hrs, 37°C) or heat inactivation by boiling for 30 min. Thus, given the above results, conditioned medium will be systematically subjected to anionic column purification and the resulting eluate will be used in our assay. This should not present a major increase in the work load since such a chromatographic purification

together with the ensuing concentration steps will only extend the conditioned medium processing by 2-4 hours.

The Henderson laboratory has considerable experience in using MNs as tools for high-throughput screens. We are in the process of learning some of these techniques for SA I. Some examples of these key techniques and results are shown in Figure 4: **A**. The Flash Cytometer provides an unbiased measure of survival and growth of all of the neurons in the culture. Complete well of a 96-well dish seeded with primary MNs, labeled using the fluorescent vital dye AM-calcein, and imaged at high-throughput using the Flash Cytometer. A negative image is shown for ease of visualization at low magnification. **B**. Screening using primary motor neurons can allow for identification of novel compounds with clinical relevance for ALS. Dose-response curve for survival

Figure 4. High-throughput screening technologies



effect of TRO19622, the first ALS drug candidate to be identified by high-throughput screening on primary MNs. Survival (mean \pm SEM; $n=8$ wells) is expressed as a survival ratio. A value of zero corresponds to the negative control (survival without trophic factors) and a value of 1 corresponds to the positive controls (survival with an optimal cocktail of trophic factors). **C**. Axon outgrowth can be reliably quantified in a fully automated manner. Close-up image of a fraction of a well in a 96-well dish containing ES-MNs expressing Hb9::GFP grown on monolayers of CHO cells. Live cells are imaged through the monolayer using the Flash Cytometer. False colors indicate the automatic identification of individual neurons by Metamorph,

which calculates neurite outgrowth parameters for each. **D**. Axon outgrowth quantified automatically is a highly reproducible, dose-sensitive readout. Dose-response curve for neurite outgrowth determined during a screen currently underway in the Henderson lab in collaboration with Dr. B. Stockwell. ES-MNs were grown on monolayers of CHO cells stably expressing the growth-inhibitory myelin component MAG and imaged as in D. Axon outgrowth (mean \pm SEM; $n=8$ wells) was measured in untreated cultures ("Control"), in the presence of forskolin and IBMX ("F+I"), and in the presence of

decreasing concentrations (10 to 0.63 μM) of the ROCK inhibitor Y-27632. Axonal length measurements are highly reproducible and dose-sensitive.

As a first step toward developing our cell-based model for high-throughput studies, we have successfully adapted it to a 96-well-plate format. For this work, 2,500 ES-MNs were plated per well in 96-well plates in which half of the wells contained confluent wild-type astrocytes and half contained confluent mutant SOD1 astrocytes. The plated ES-MNs were monitored using the Flash Cytometer at 1, 5, 7, and 8 DIV. The GFP⁺ MN counts obtained using the software TINA showed that the survival of ES-MNs grown on SOD1^{G93A} astrocytes decreased over time to 55% of that of their counterparts grown on wild-type astrocytes by 7 DIV, and did not decrease further thereafter. These results are in agreement with those we obtained previously in the 24-well-plate format (32, 33). This finding was replicated when 2,500 ES-MNs per well were seeded in 96-well plates in the presence of media conditioned with either wild-type or SOD1^{G93A} astrocytes. After 7 days exposure to mutant conditioned medium, a ~50% ES-MN death was measured, confirming that this time point is appropriate to evaluate the effects of the small molecules. However, regardless of the genotype of the conditioned medium, the number of surviving ES-MNs after 7 days was half that of those cultured on astrocyte layers. To circumvent this issue, we thought to plate a higher number of ES-MNs ranging from 5,000 to 10,000 per well. This showed that, at the highest density we tested thus far (i.e. 10,000 per well), the number of surviving GFP⁺ ES-MNs after 7 days of exposure to conditioned media was twice that previously observed with a density of 2,500 ES-MNs per well. While increasing the plating density did increase the dynamic range (i.e. positive control signal [mean MN number_{V5}] – negative control signal [mean MN number_{DMSO}]) of the assay in absolute numbers of MNs, it did not change the signal-to-background (S/B) ratio (calculated here as mean MN number_{V5} / mean MN number_{DMSO}) which remained at ~2. While this step does improve the assay somewhat by providing a larger number of countable ES-MNs, clearly we still had to try to increase the S/B ratio to at least 3. Indeed, this will put us in a situation in which, if we obtain a reasonable coefficient of variation (CV; SD/mean), we can expect to attain our desired statistical power of ~0.80; see below for further discussion on CV and power calculation.

In reviewing the inter-well variation, we found that the mean CV per line and per column in this pilot experiment was $5 \pm 1\%$ at 1 DIV regardless of whether the plating density was 2,500 or 10,000 per well, confirming the efficiency of our plating technique and the possibility of plating ES-MNs at high density. But at 7 DIV, the mean CV was $15 \pm 1\%$ which exceeds the limit of acceptability to ensure the proper optimization of the assay (32-34). Indeed, in reviewing Figure 1 of the paper by Sui and Wu (34), it clearly appears that a CV of 15% would preclude reaching a power of ~0.80 and a Z'-factor ≥ 0.5 which, according to the National Institutes of Health Chemical Genomics Center (<http://ncgc.nih.gov>) and the National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (<http://nsrb.med.harvard.edu>), indicate proper assay optimization. Among the different ongoing technical standardization and optimization steps, we are working on assessing and, if needed, minimizing variations due to the positional effects (e.g. edge effect) and the plate-to-plate variations. More importantly, note that thus far, we have used

unpurified ES-MN cultures which are made of 40-50% MNs and 50-60% of other cell types including astrocytes. We surmise that this cell mixture in our assay that contributes to the inter-well variability. As shown above, we can now obtain pure ES-MN cultures thanks to the use of our validated FACS protocol. We are now evaluating the gain on the inter-well variation that we can expect from using pure ES-MN cultures. It is our belief that the different remaining optimization steps will bring us close to a CV of $\leq 10\%$, which is our goal.

To guide us in this optimization of the assay, we have followed the value of the Z'-factor (32) and while this factor does not rely on distributional assumption, it best reflects the quality of the assay under normal distribution (33). We subjected our data for the positive and negative controls, which are used to calculate the Z'-factor, to a Kolmogorov-Smirnov normality test. Thus far, neither the data for the positive nor for the negative controls diverge significantly ($p > 0.05$) from a normal distribution. In our first set of experiments for this part of the work, we obtained a Z'-factor of -0.06, which is a sign of a poor quality assay. In analyzing these disappointing first results, we found that one of the main reasons for this poor Z'-factor was the narrowness of the dynamic range and the low S/B ratio of our current assay. Indeed, the difference between our positive and negative control (ES-MN numbers in wild-type and mutant conditioned medium, respectively) was only slightly superior to the value of the negative control itself (192 vs. 186 cells) which gave an S/B ratio of ~ 2 . As shown above, we have recently found that processing mutant astrocyte-conditioned media through a Q Sepharose™ XL strong anion exchange was capturing the toxic activity, hence enabling us to concentrate it to increase MN toxicity at 7 DIV. With this chromatographic step, the S/B ratio jumped from ~ 2 to ~ 3 which is at least where we wish to be with the S/B ratio to obtain a high quality assay that operates at an excellent statistical power of 0.80. Now, if we recalculate the Z'-factor with our current CV of 15% and S/B ratio of ~ 3 , we obtain a Z'-factor of 0.23 which is a huge improvement of our assay, even if it is still below our desired (and recommended) target of 0.50 as discussed above.

Under these last set of values, we can also calculate the power of the assay (i.e. probability of declaring active compounds as hits) using the R free software (<http://www.r-project.org/>). However, first we should determine whether we are dealing with an assay where the data for the positive and negative controls exhibit constant SDs or rather constant CVs since, as reminded by Sui and Wu (34) these define two very different models of power calculation. Not surprisingly for a cell-based assay, we found that the SDs were proportional to the means: the higher the signal, the higher the SD. Thus, the SDs for the positive controls were higher than those for the negative controls, but the CVs were the same. Consequently, despite the fact that the SDs did not grossly diverge from each other, we thought it safer to use the stricter model of power calculation based on constant CVs. For this simulation, we have considered that an active compound must inhibit MN death by at least 50%; meaning the number of GFP⁺ MNs is increased halfway toward the positive control, V5. We arbitrarily chose 50% inhibition for this simulation as it represents a likely meaningful magnitude of MN death inhibition in our cell assay for this primary screen of small molecules. We hope that our lead compounds will inhibit MN death well beyond 50% (e.g. 75-80%). However, to declare a compound as a hit based on the fact that it exerts 50% rather than 75% inhibition of MN death, represents a more stringent situation for which the assay must

be perfected further to still maintain a power of 0.80. Thus, this arbitrary 50% inhibition level represents, in our opinion, a reasonable compromise between neuroprotective meaningfulness and statistical stringency. Using these parameters, currently our power is estimated at ~0.21, which is insufficient. However, given the optimization steps in the works and outlined above, a simulation of power calculation shows that we can attain our goal of 0.80 with an S/B of ~3, if the current CV of 15% is reduced to ~10%. With the different improvement steps still available to us to reduce the CV, we believe that this is a reachable goal. Thus, we are confident that on completion of our assay optimization, we will have in hand a high-throughput assay ready for the proper screening of our library of small molecules.

We did encounter one problem that arose in our cell culture room. Because of temperature spikes (~90°) in our culture room, which was attributed to a malfunctioning air flow system in the building, a mycoplasma infection developed. To correct this situation, the airflow system, as well as the incubators, was sanitized. We had all of the hoods serviced and the entire room washed down. While our culture room was being cleaned and cleared of the mycoplasma infection, we did our work in the Henderson Lab, so that our work would not be compromised.

Year II (2009-2010)

Conditioned Medium Validation Studies

This year was dedicated mainly to MNs and ACM and the validation of the effect of mutant ACM on MNs. Below are the figures which layout the chronology of our accomplishments for the second year of this award. Figure 5 demonstrates how our ACMs, both WT and mutant, are produced. Basically, WT or mutant spinal cords are skillfully removed and prepared for culture as per (27). Medium from the cultured G93A mutant astrocytes is collected and centrifuged to remove the astrocytes. Medium was then used to test its toxicity on spinal motor neurons.

Astrocyte-conditioned medium

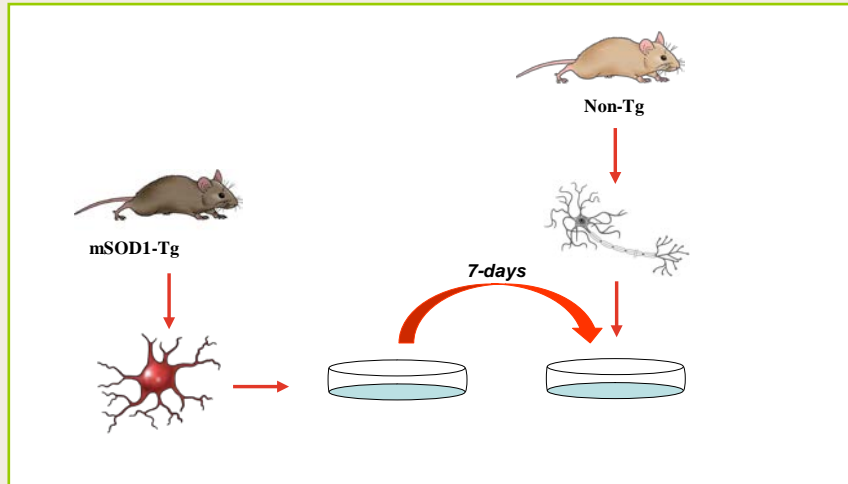
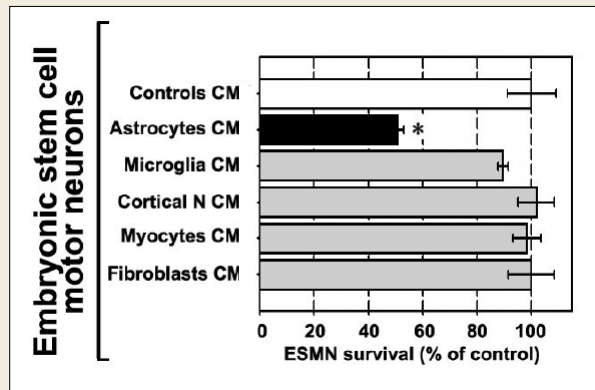


Figure 5. Composition of our astrocyte-conditioned medium.

Following the production of the ACM, our next step was to test the specificity of the mutant ACM, that it indeed is the result of astrocytes being cultured in the medium. Furthermore, we needed to test that this ACM produced from mutant astrocytes was indeed toxic and that this property was not characteristic of other types of cells, such as microglia, myocytes, fibroblasts and cortical cells. Figure 6 demonstrates that only the conditioned medium derived from mutant astrocytes is toxic to MNs and that this effect was quite similar to MNs cultured with mutant astrocytes.

Mutant conditioned medium has effects on MNs similar to mutant astrocytes



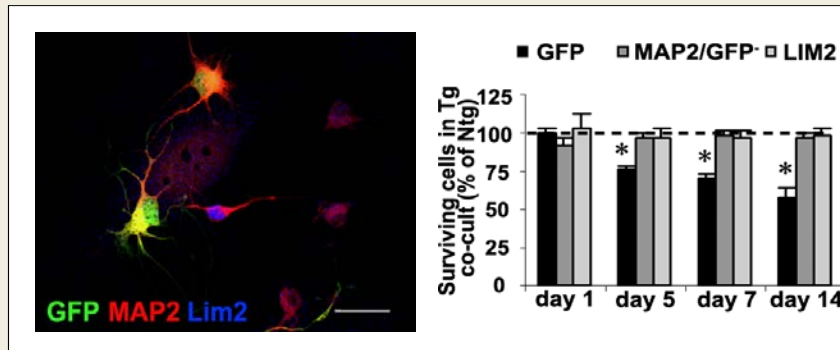
Nagai *et al.*, Nature Neuroscience, 10(5): 615-622, 2007.

Figure 6. Demonstration that media conditioned with other types of cells are not toxic to embryonic stem cell-derived motor (ES-MN) neurons.

Our next step was to assess the effect of our ACM on ES-derived MNs to determine the level of vulnerability, if any, of these cells to ACM. A significant decrease in the number of surviving ES-MN is first seen at day 5 of exposure time and, by 14 days of exposure, only 50% of the ES-MN survive. In Figure 7, it was noted that ES-MN vulnerability to mutant ACM is time-dependent in that the longer the exposure time, the greater the number of ES-MNs that die. Thus, ES-derived MNs behave like primary MNs when it comes to the toxicity of mutant astrocytes to MNs.



Embryonic stem cell-derived MNs (ES-MNs) are selectively vulnerable to mutant SOD1 astrocytes (or ACM)

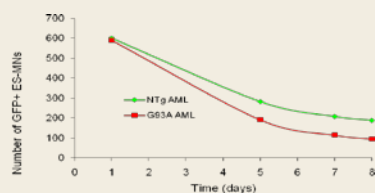
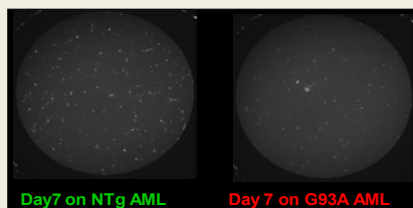


Nagai *et al.*, Nature Neuroscience, 10(5): 615-622, 2007.

Figure 7. ES-MN vulnerability to mutant astrocyte-conditioned medium is time-dependent; the longer the exposure time, the greater the number of ES-MN that die.



Recapitulate astrocyte toxicity with ES-derived MNs in 96-well plates



- To guide us in the optimization of the assay, we use Z'-factor:

$$Z' = 1 - \frac{(3 \times \text{STD positive Ctrl} + 3 \times \text{STD negative Ctrl})}{(\text{Mean of posit Ctrl} - \text{Mean of negat Ctrl})}$$

- “-0.08” → a poor quality assay (excellent assay $Z' \geq 0.5$).
- Low Signal/Background (S/B) ratio (~2-fold)
- High mean CV per line and per column at 7 days (25 1%)

Figure 8. Minimization of the conditioned medium toxicity assay.

The *Z'-factor* is a measure of statistical effect size. It has been proposed for use in high-throughput screening to judge the response of a particular assay. This factor is

important because of the screening of large numbers of samples. What is important here is the size of the Z'-factor, the higher this number, the better the assay. Thus, part of our miniaturization of our assay was to increase the Z'-factor number, and to increase the signal to background ratio (Figure 8).

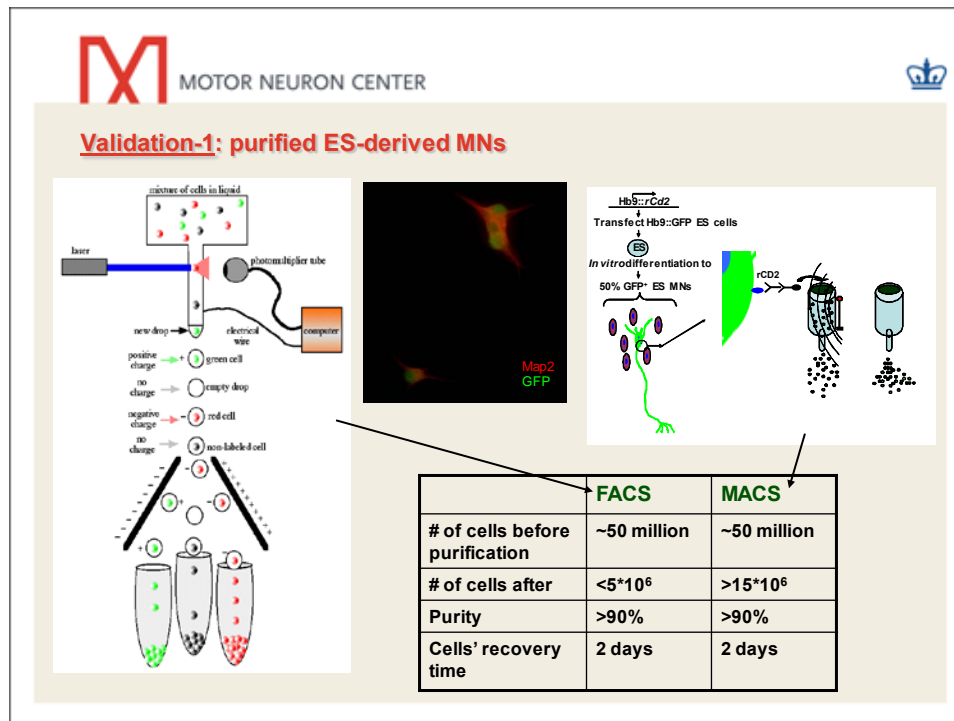


Figure 9. Which sorting system is better here? MACS yields 3X the number of ES-MN as FACS.

Part of this year's work was to decide on which cell sorting system (MACS, magnetic activating cell sorting or FACS, fluorescence activating cell sorting) was better in sorting ES-MNs for our assay. While both sorting systems produced 90% purity in sorting the cells, the MACS sorting system was far superior to the FACS in producing cell numbers. (Figure 9). Thus, it was determined that MACS is the best sorting system for our work

Time course of purified ES-MN survival over 7 days

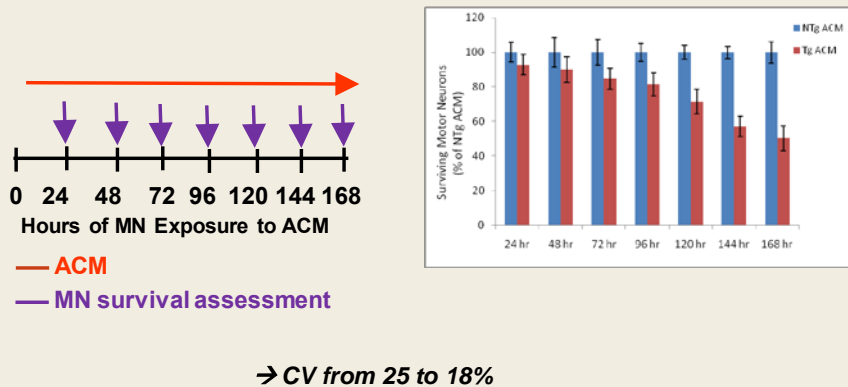


Figure 10. ES-MN survival in the face of constant exposure to astrocyte-conditioned medium.

We also compared ES-MN survival to primary MN survival in mutant ACM for the validation of our assay and here, we show that MNs and ES-MNs (Figure 11) exhibit the same level of toxicity.

Validation-2: stability of the toxic activity

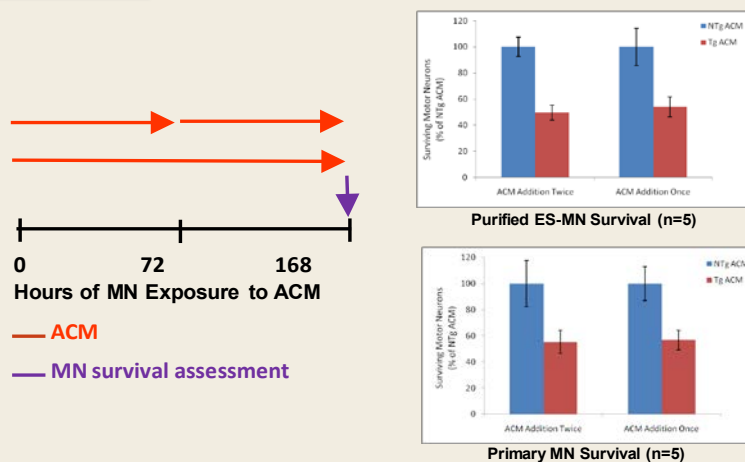


Figure 11. Primary MN and ES-MN exhibit the same level of survival or the same level of toxicity in astrocyte-conditioned medium.

Signal to background (S/B) ratio is defined as the ratio between the background noise and meaningful information. Improvement of this number improves the value of the assay. So, part of our work on this project was to increase the signal to background ratio by attempting to concentrate the toxic activity of the mutant. Using column chromatography, we increased the S/B ratio of the mutant ACM from 2 to 3 and at the same time, pinpointed the toxic activity to less than 30kd.

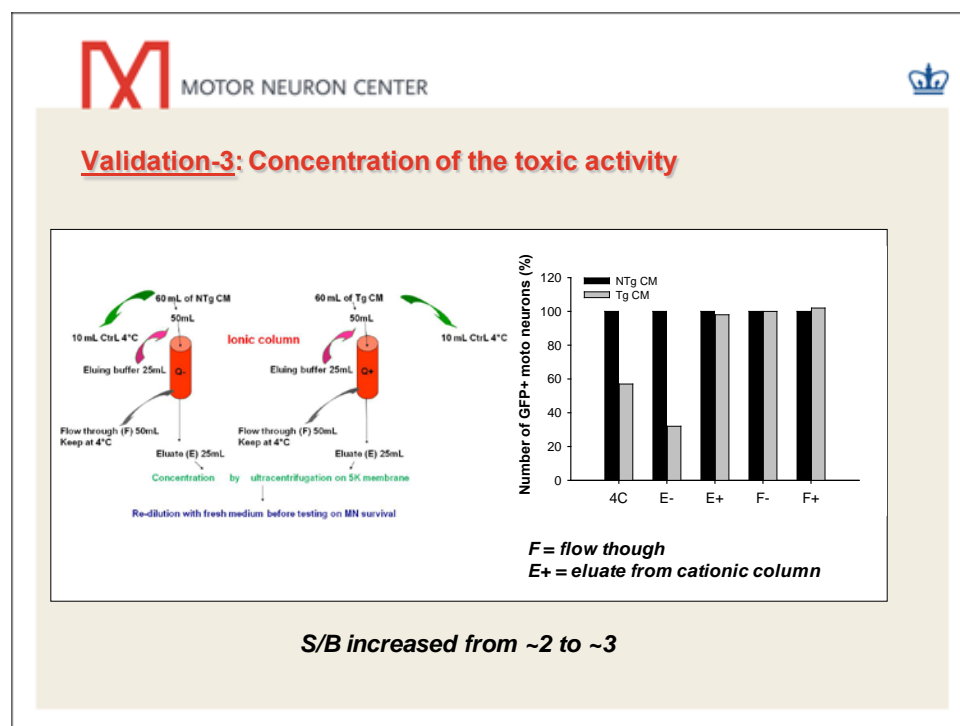


Figure 12. Concentration of toxic activity using column chromatography.

We next tackled the coefficient of variance (CV) issue in relation to plating density. CV is the expression of standard deviation in percent. And, the smaller this number, the better. With our high throughput screening method, we have reduced the CV to 18%. We did note that plating density and CV were related. Figure 13 lays out the results of our efforts on studying plate density for our high throughput assay.

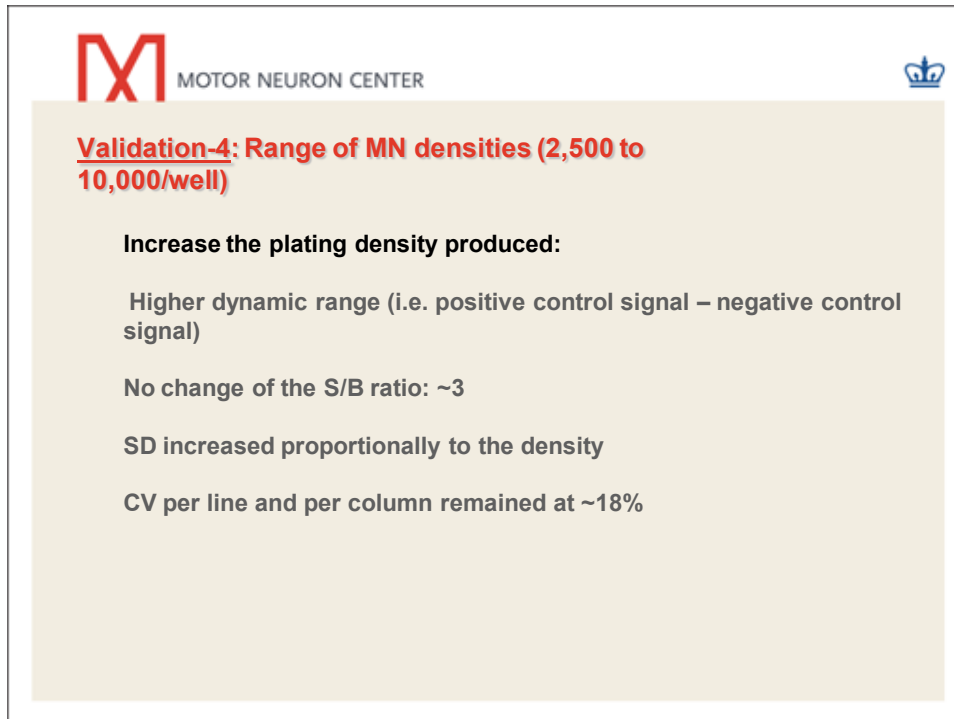


Figure 13. Results of MN density per well.

High throughput screens (HTS) analyze large chemical libraries (over 50,000 compounds) for possible hits that might be useful in the treatment of disease. On the other hand, the low throughput screen is a screening process to probe the possible mechanisms of action in the production of a disease state. In our investigations of the possible mechanisms involved in the death of spinal cord motor neurons, we noted that certain pathways more than others were involved here. Thus, to pinpoint possible mechanisms in ALS, we used our ACM-ES-MN co-culture system to narrow our focus on the etiology of the disease. Thus, Figure 14A demonstrates that JNK inhibition, but not p38, caspase-8 or nNOS inhibition, rescues spinal MNs from death. Furthermore, we also demonstrated that Bax ablation, but not that of Bim, Bak or p53, blocked MN death in our co-culture system (Figure 14B). Type of cell death is an issue in ALS as knowing the type of cell death can possibly dictate the treatment. Thus, our co-culture system was of use here in that we exposed it to Necrostatin, an inhibitor of programmed necrotic cell death. In Figure 14C, we demonstrate that Necrostatin inhibited MN death in our ALS co-culture system. This experiment suggests that spinal motor neurons may die by a programmed necrotic form of cell death. Finally, we used our co-culture system consisting of ES-MNs and mutant astrocytes in growth factor withdrawal studies (Figure 14D) and noted that growth factor supplementation can rescue ES-MNs.

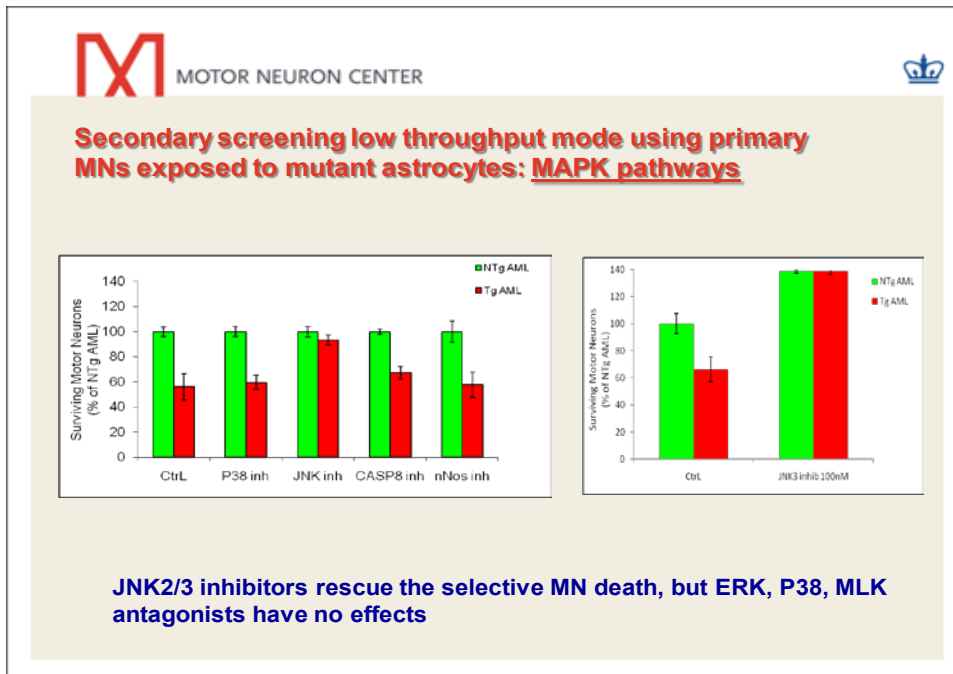


Figure 14A. JNK inhibition but not p38, caspase-8 or nNOS inhibition rescues spinal motor neurons from death following exposure to mutant SOD1 astrocytes.

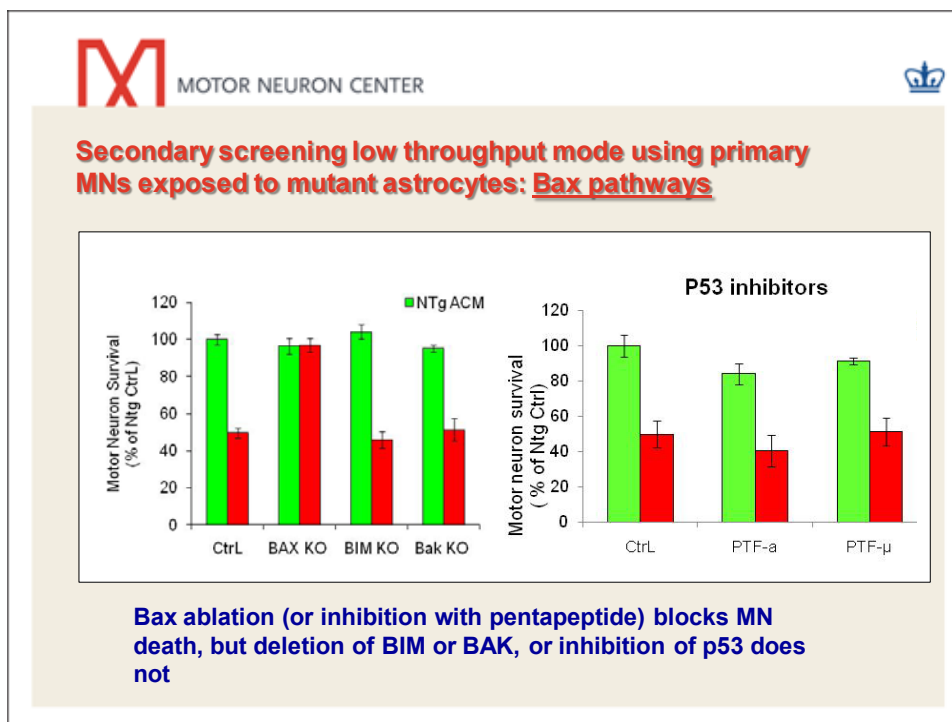


Figure 14B. Low throughput secondary screen targeting the Bax pathway using primary MNs in the presence of mutant astrocytes.

Figure 14C. Low throughput screen using Necrostatin in the presence of mutant astrocytes to prevent MN death.

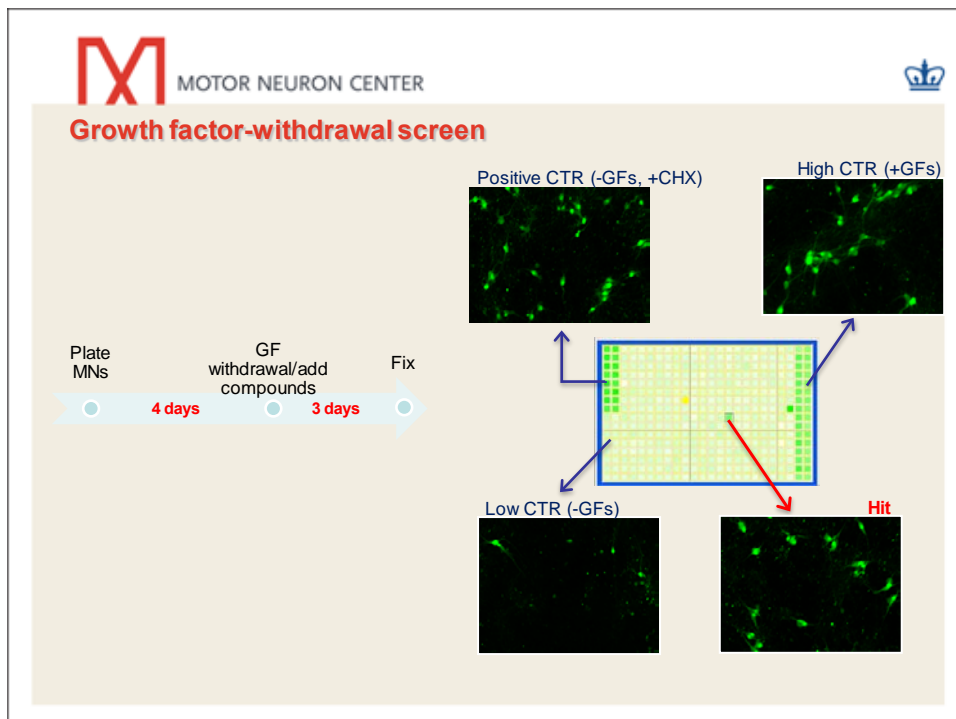
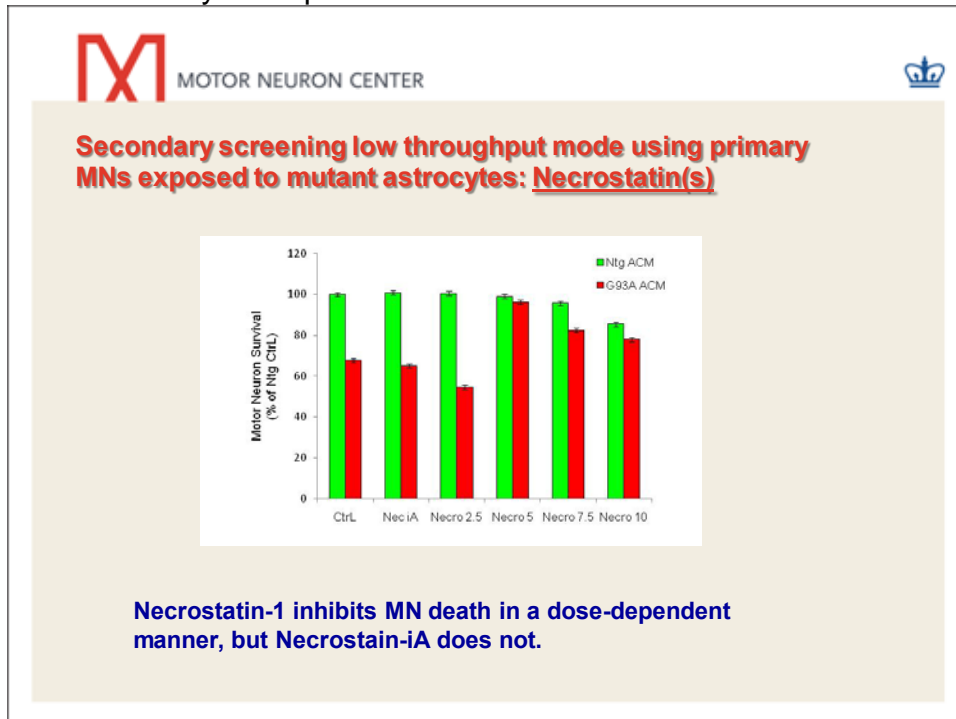


Figure 14D. Growth factor withdrawal/supplementation studies with ES-MNs and mutant SOD1 astrocytes.

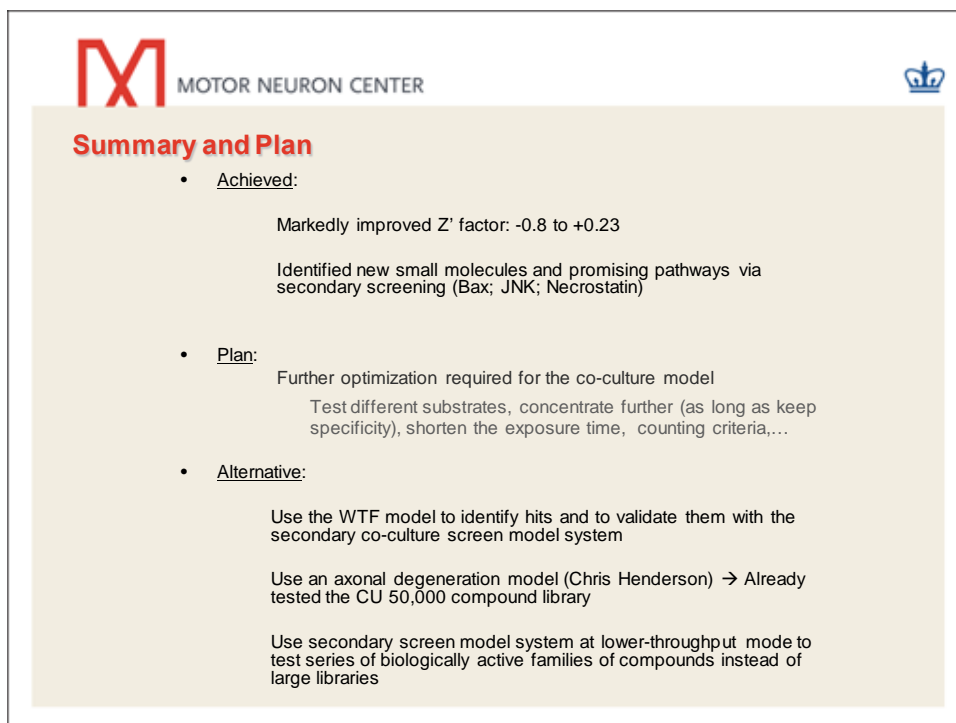


Figure 15. Summary of what was achieved during the 2009-2010 time period.

Year III (2010-2011)

The overall goal of this project is to identify small molecules that could be useful for the treatment of ALS. To date, as mentioned above, the only two treatments that have been approved for this debilitating disease are mechanical intervention and riluzole. However, these are of very limited use. In our efforts to improve the high throughput assay, we have found that SOD1 mutant astrocytes produce a factor that is toxic to spinal MNs [12] and to ES-MNs. So, rather than trying to culture spinal MNs from spinal cords of mutant SOD1 mice, we found that we could use these ES-MNs instead and they work just as well. Therefore, using the toxic effect of mutant SOD1 astrocytes and the availability of ES-MNs, we improved further our co-culture system that is to be used extensively to screen small molecules of potential interest. Below are our accomplishments for year 03 of this extensive project.

Further improvement of the Z' score

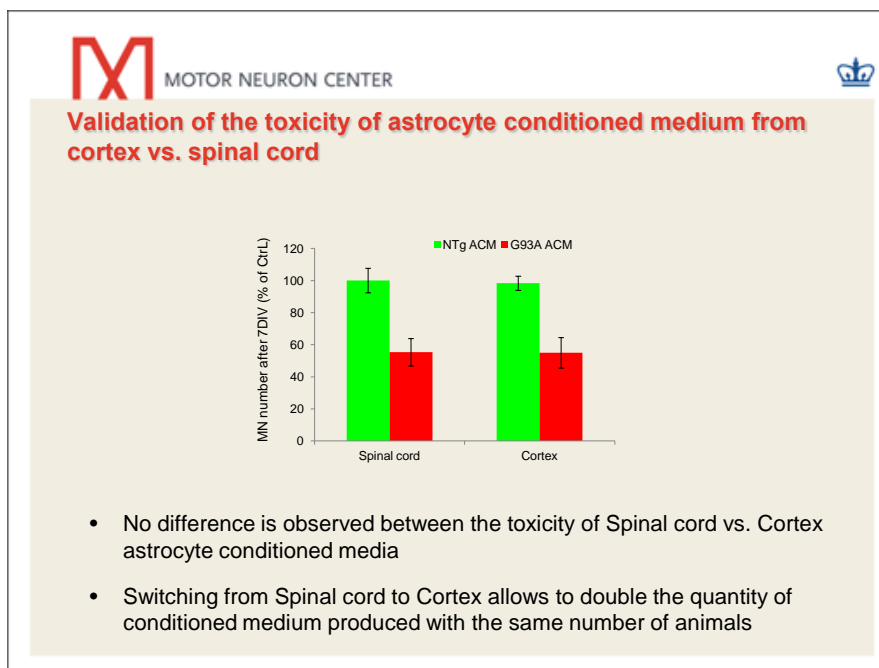
At the time of our last progress report, our Z'-score was ~0.23 which is below the score needed for an optimal assay. Given this fact, we pursued **SA I** by spending more time on further validation of our assay. To improve the Z- score, we decided to test a variety of plate coatings and ES-MN seeding densities. After testing a number of combinations, we found the right combination of plate coating and seeding density which increased the signal to noise ratio from 2 to 2.6 and, even more importantly, lowered the coefficient of

variability from 25 to 9% at 7 DIV. With these improvements, our Z'-score shifted from 0.23 to 0.30, which is considered acceptable, but still not optimal, for a cell-based assay.

Increased production of conditioned medium

As indicated in our previous progress report, wild-type MNs can be killed merely by exposure to culture medium conditioned for 7 days with mutant astrocytes (ACM). However, one major drawback to our screening was the production of sufficient amounts of ACM for this screening. We have only used astrocytes from spinal cords. To try to overcome the insufficient amount of ACM production, we thought to use larger regions of the nervous system such as cortical areas. Thus, we purified astrocytes from this region and used cortical astrocyte cultures to produce larger amounts of ACM. As shown in Figure 16, we saw no difference in terms of toxicity towards MNs when we used ACM produced from mutant astrocytes purified from spinal cord or cerebral cortex. This simple change allows us to double our production of ACM.

Figure 16: Set up to improve production of the astrocyte conditioned media

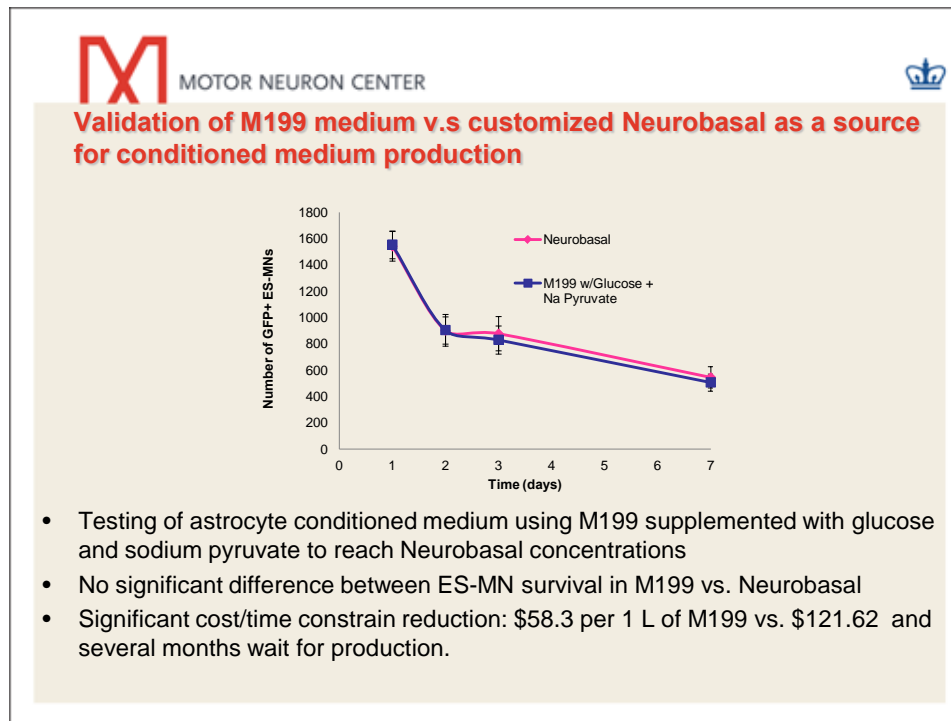


M199, a new and less expensive culture medium

Because our initial culture medium was the Neurobasal medium, we initially purchased customized non-fluorescent Neurobasal medium for our work. While this was very convenient, it presented a serious budgetary burden. Thus, we thought to test a series of non-customized, non-fluorescent culture media. After a number of testings, we demonstrated that, if we use M199 medium supplemented with glucose and pyruvate,

the kinetics of MN death, in response to toxic ACM, was identical to that obtained with the non-fluorescent Neurobasal medium (Figure 17). This move proved to be quite advantageous as it cut, by half, the cost of culture medium required for this work.

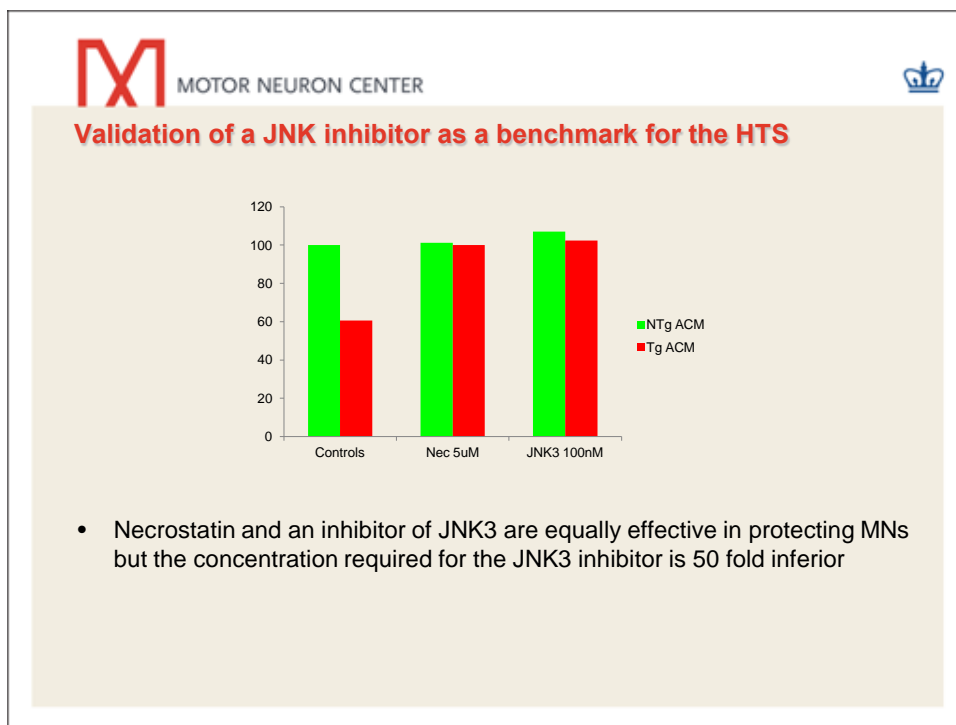
Figure 17. Testing of new culture media



New benchmark compound

Another economic issue was the cost of our initial commercial benchmark compound Necrostatin. Thanks to the low-throughput screen that we have performed for the past few years in our laboratory, we found that JNK inhibitor 1 (JNK1) protected purified ES-MNs against mutant ACM. We compared, side-by-side, the beneficial effects of both Necrostatin and JNK1 (Figure 18). This experiment showed that the level of protection obtained at 7 DIV using 5 μ M Necrostatin was equal to that obtained with 0.1 μ M JNK1. This meant that JNK1 could be used in our high-through screen (HTS) at a concentration 50 times lower than Necrostatin, which allows us to make significant savings.

Figure 18. Comparison of benchmarks



Library composition: (Figure 19).

As indicated above, given the delay in validating our cell-based assay, we decided to scale down our library of small molecules. With the help of our collaborator, Dr. Brent Stockwell, we have selected a set of 2,000 biologically active, non-redundant, non-overlapping compounds. All of these 2,000 biologically active compounds have a wide range of structural diversity and have properties favorable to effectively crossing the blood-brain-barrier, as assessed by *in silico* algorithms. Among these, 80% are FDA-approved drugs or natural compounds and each compound has a minimum purity of 95%. One thousand molecules (50%) are drug components, of which 99% of these are off-patent; 600 (30%) are natural products which have been selected for their chemical class and structural diversity; and 400 (20%) are other bioactive components such as inhibitors, blockers and toxins.

New timeline

With the different additional validation steps detailed above, we believe our validation phase was completed in mid-spring 2011. Since then, we have started to screen the compounds of the new library outlined above. We expect that we will complete the testing of these 2,000 compounds early in 2012. From now until early summer 2012, we expect to validate our identified hits by performing dose-response curves in ACM/ES-MN cultures and to confirm ACM toxicity in primary spinal MN cultures.

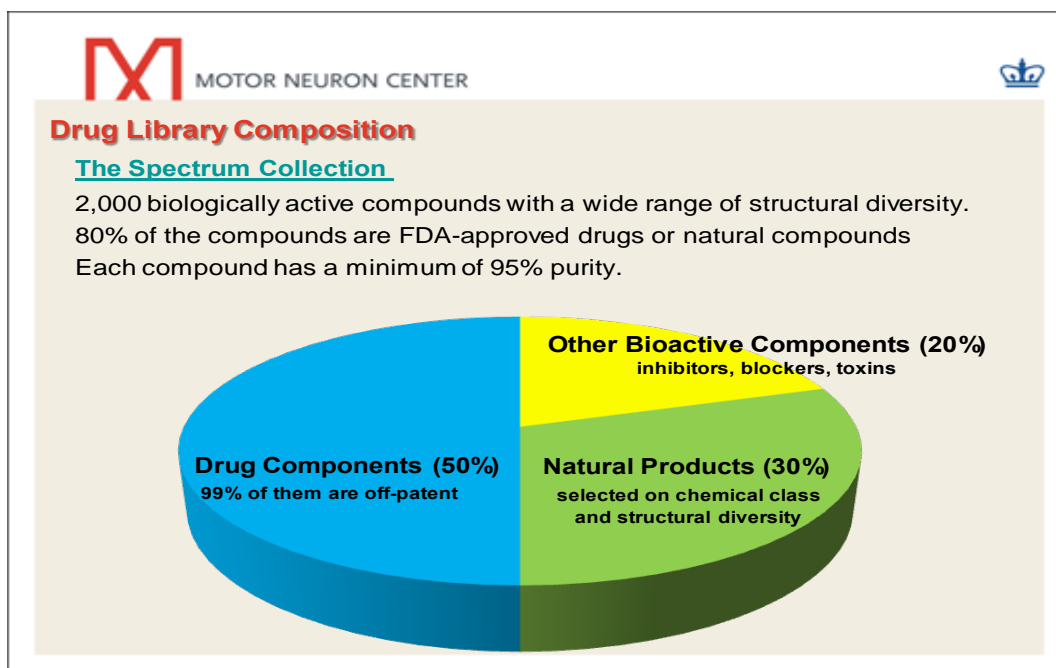


Figure 19. Drug Library Composition: drugs that are off-patent (50%), bioactive compounds (20%) and natural products (30%). Most of these compounds have FDA approval, are about 95% pure and are available for testing as possible disease treatments.

Concomitantly, we will pursue our low throughput screens of the MAPK and the BCL-2 pathways using primary MNs. By exposing primary spinal MNs to mutant ACM, we have previously found that, in contrast to JNK inhibition, inhibition of other stress kinases, such as P38 and ERK, or inhibition of nNOS and caspase 8, in MN degeneration, did not exert any protective effect on MNs upon exposure to mutant ACM.

Year IV (2011-2012)

Here, we concentrated on the actual performance of the high throughput screen. Figure 20 is a summary of the improvements made in our assay. These include further improvement of the Z factor, further improvement of the signal to noise ratio, a decrease in the coefficient of variance and an increase in plate MN numbers in plate seeding. Following Figure 20, Figures 21A and 21B represent a visual of actual high throughput screen components and workings in our co-culture system.

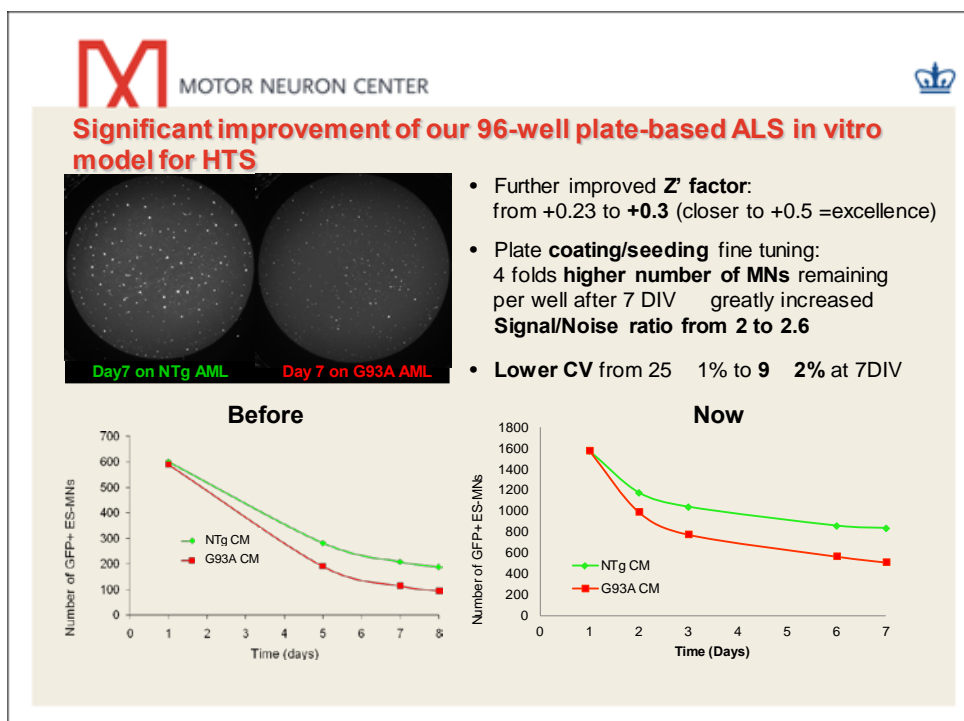
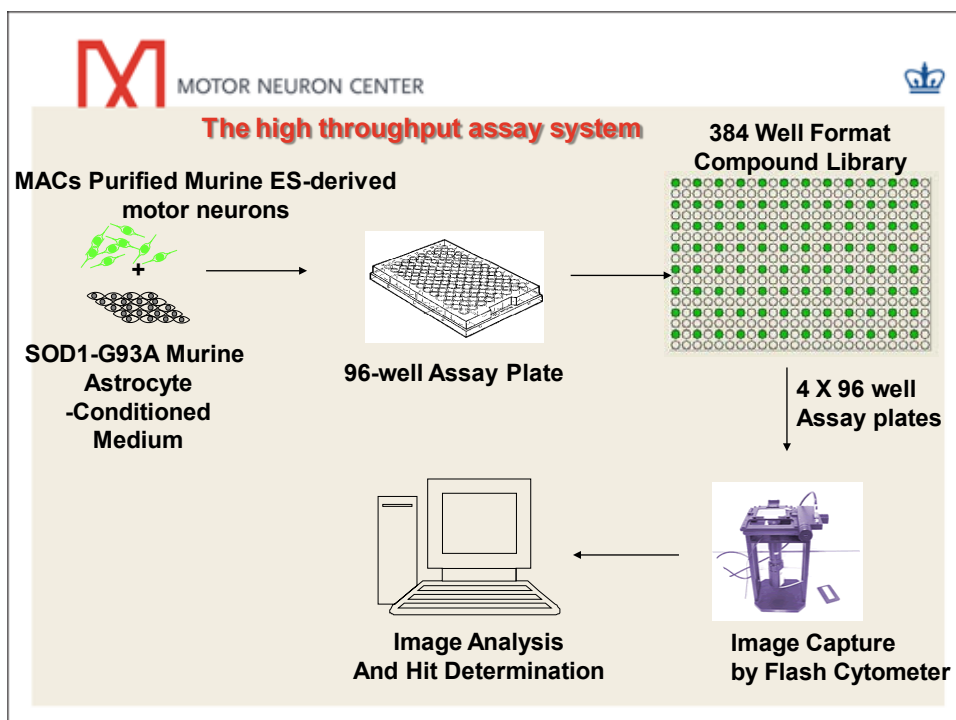


Figure 20. Summary of the Improvements to our invitro high throughput screening method.



Figures 21A and 21B (below) display the components of the high throughput screen and the plate mapping that we will be using in our method

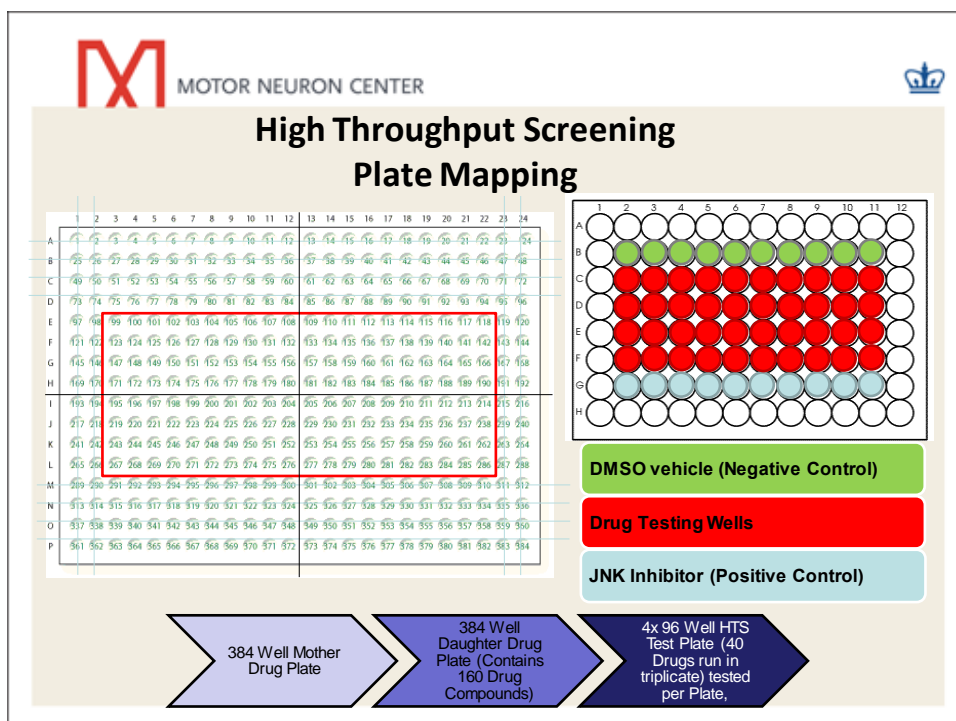


Figure 21B. How the throughput screening plate is laid out.

Thus far, we have identified 108 hits out of the compounds that were screened as possibilities for ALS using our high throughput screening co-culture system. While 7% of these 108 compounds are bioactive compounds, 46% are off-patent and 47% are natural compounds (Figure 22). These compounds (Figure 23) represent a range of different classes of compounds such as antioxidants, anti-inflammatories, antibacterials, agonists, antagonists, etc and will move to the next level of testing. Figure 24 lists some of the compounds that have passed the first screen and have been moved to the next level of the screening process,

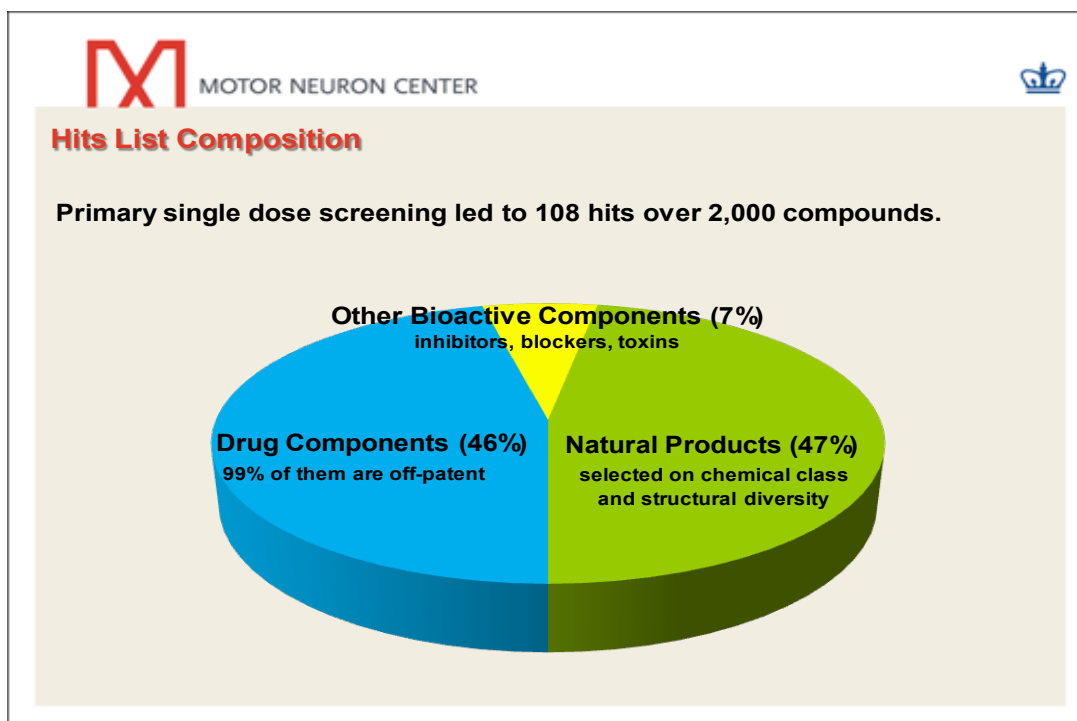


Figure 22. Composition of the 108 hits from the initial high throughput screen.

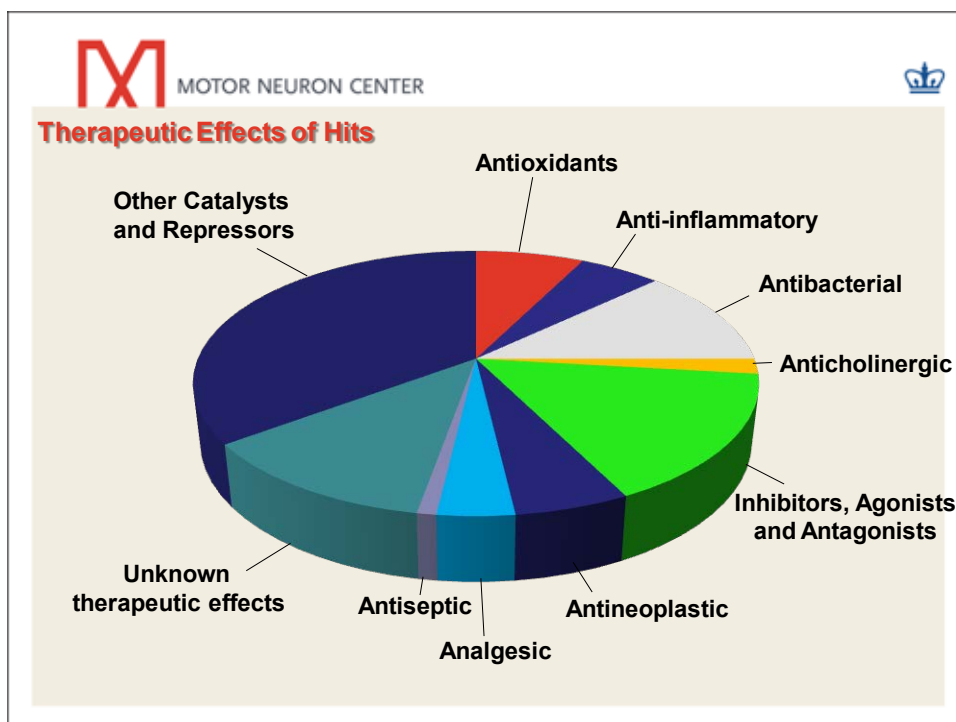


Figure 23. Modes of action of the 108 hits by groupings.

Figure 24. Some of the compounds that have passed the first screen and have moved to the next level: dose-response validation

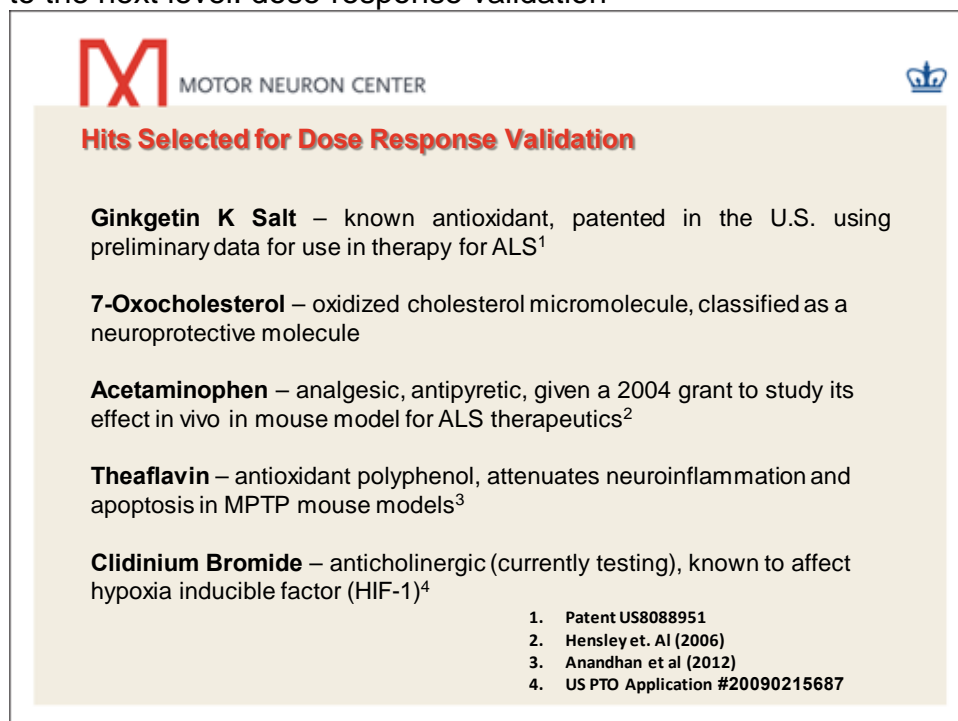


Figure 25A-25E. Dose-response validation for the selected hits in Figure 24. Figure 25A

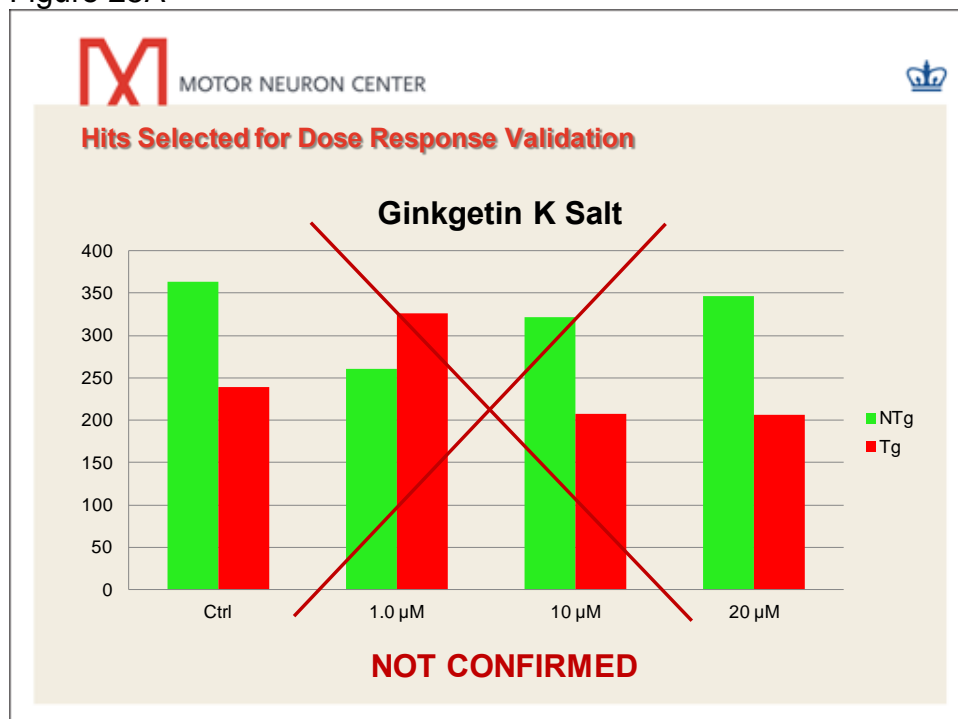


Figure 25B.

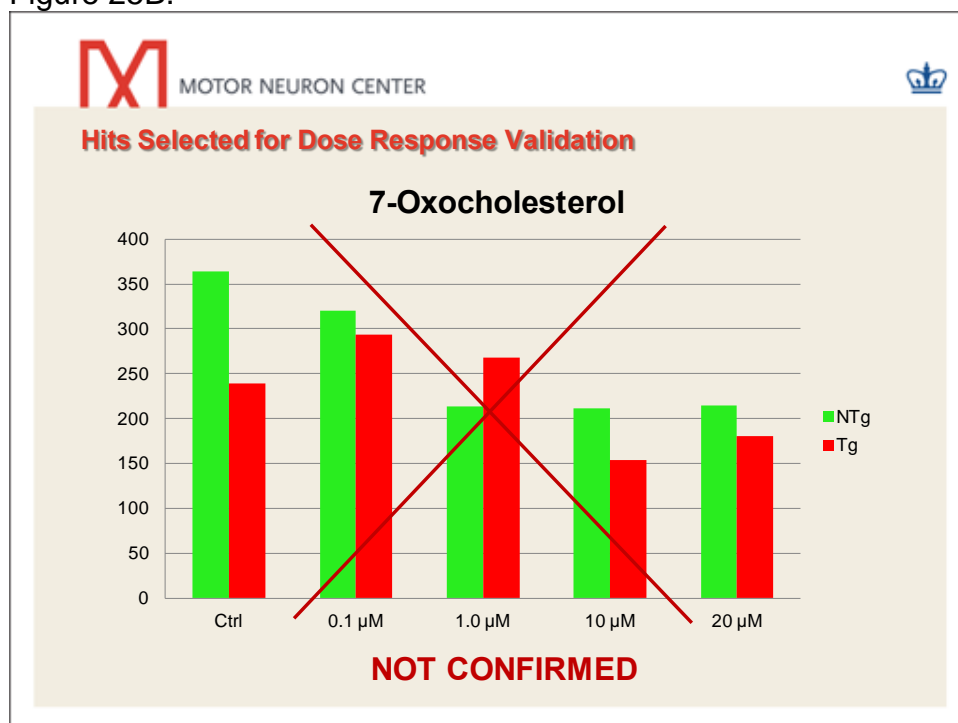


Figure 25C.

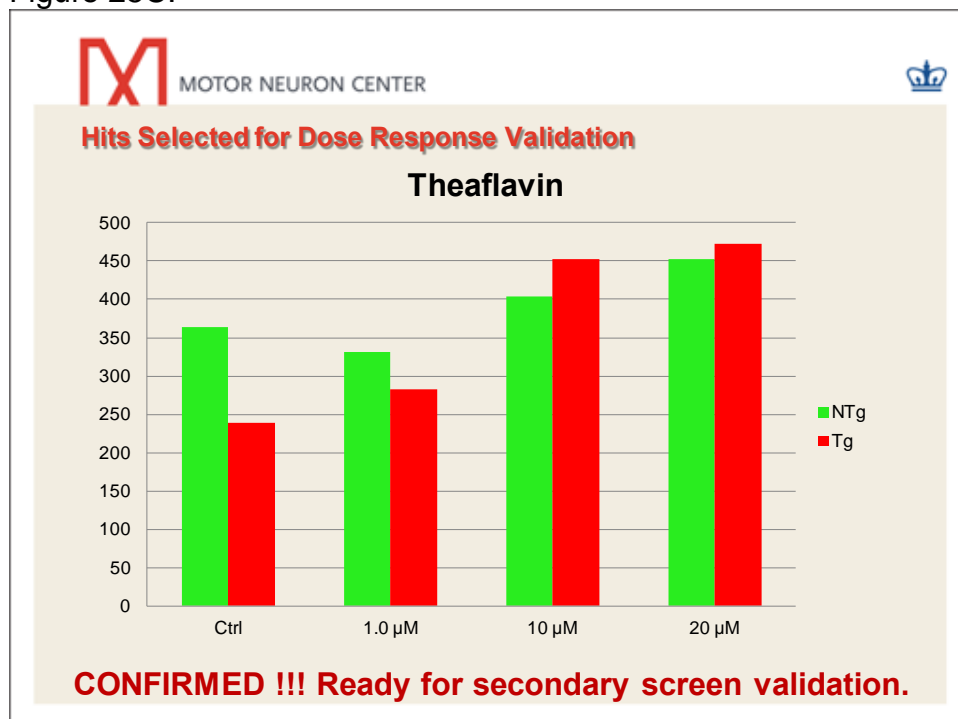


Figure 25D.

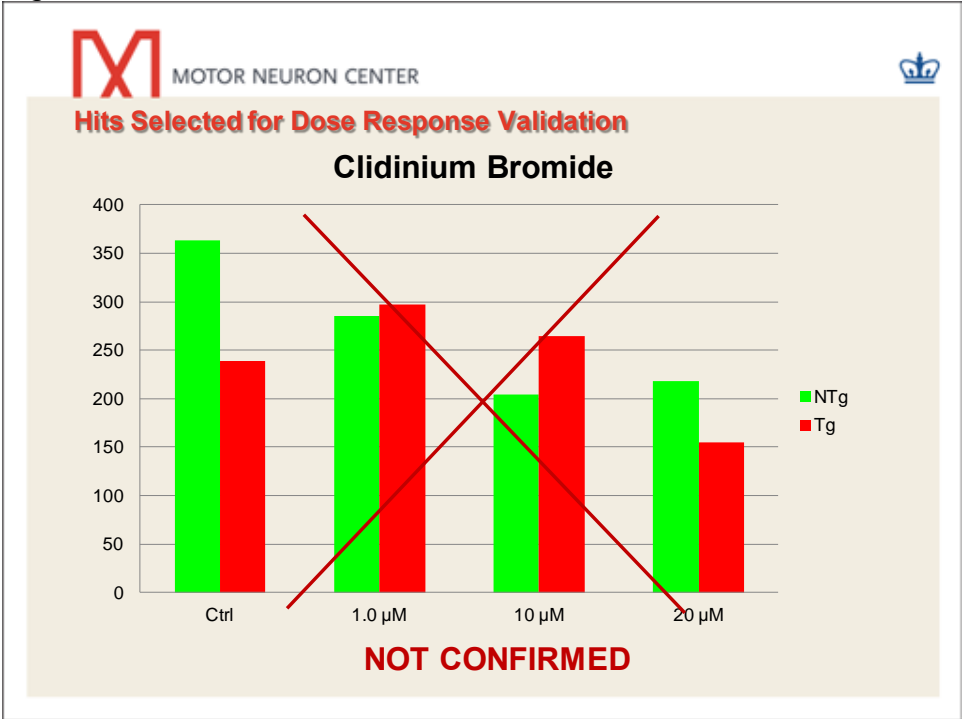
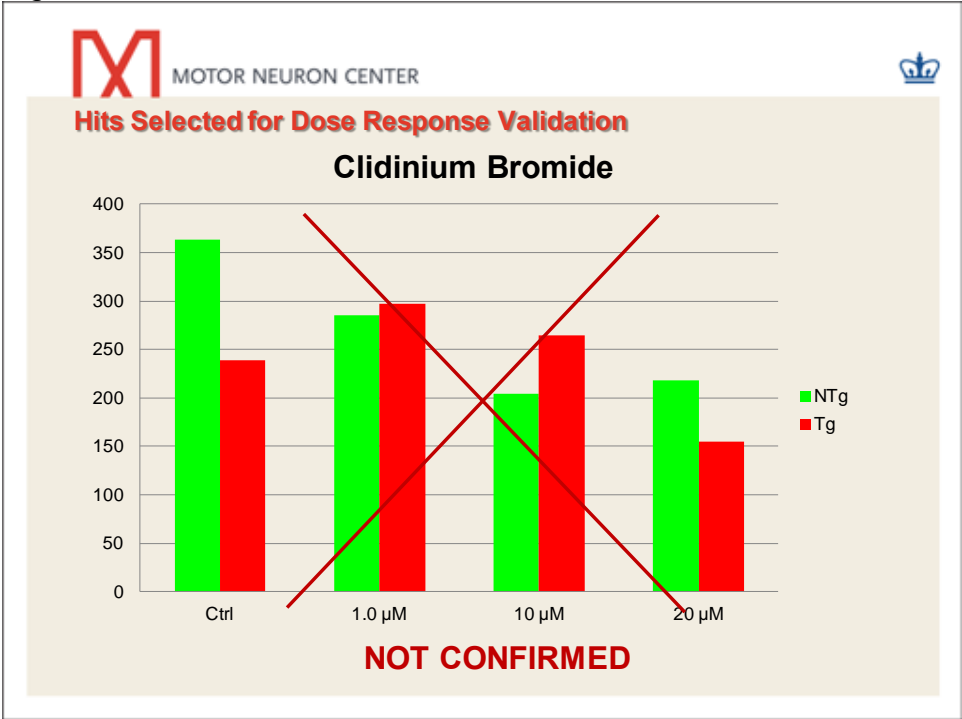


Figure 25E.



Our high throughput screening assay now seems to be heading toward being quite a decent assay to screen compounds at a much faster rate than is done at present. ALS is a debilitating disease and quality of life is greatly diminished. So, any means to get closer to some kind of relevant treatment is a plus. Thus, in our proposed screening assay, we use a co-culture system of ACM and ES-MNs. And, we have linked our co-culture system to a high throughput robot for faster throughput. The assay itself is basic as we are simply looking at MN death in the presence of ACM. For the assay itself, we have improved the Z'-factor. And, although our Z'-factor is not ideal, 0.5-1.0, maximum being 1.0, it is headed in the right direction as we have improved it from 0.23 to 0.3; 0.5 is considered ideal. Other improvements to our assay were a four-fold increase in plate seeding numbers as the four-fold increase increased the number of surviving ES-MNs in the assay. Finally, we increased the signal to noise ratio from 2 to 2.6 which increased the level of detectability in our assay. Thus, even though we will be constantly working to improve our assay, we believe that it is indeed ready for use in a high throughput situation for drugs that may be helpful in the treatment of ALS.

Year 04 (2011-2012)

Although we continue our efforts to refine our high throughput screen by working to increase the Z'-factor to at least 0.5 and to maximize the use of the miniaturized assay plate, we also continue to hunt for the cause(s) of ALS and for some cure for this debilitating disease. Thus, part of this 4th year was spent in setting up human astrocyte and human MN cultures from ALS spinal cord and from spinal cords from other diseases such as COPD, Alzheimers Disease (AD) as well as controls. Cells were cultured using the same procedures that were used for the mouse astrocytes and MNs. Our yields of the cells were good and we found no morphological differences between the human ALS and control astrocytes and MNs and no differences in the survival of these cells over several weeks between these same two groups of human tissues (Figure 26). Furthermore, in culturing human MNs with human ALS astrocytes, we found that these human MNs were indeed vulnerable to the medium conditioned with ALS astrocytes. No such situation occurred with MNs subjected to control ACM as evidenced in Figure 27. Moreover, in our co-culture system composed of human ACM and human MNs, JNK inhibition prevented the death of the MNs in human ACM (Figure 28). While these results are quite encouraging, these are early studies and much more work still needs to be done.

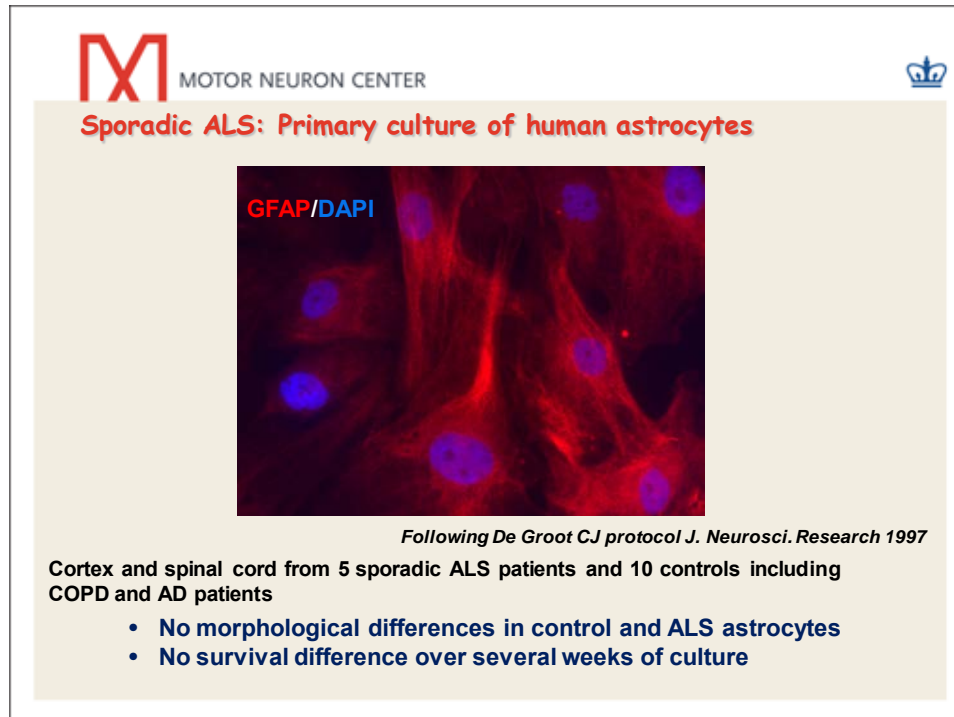


Figure 26. Primary culture of human motor neurons with human mutant astrocytes.

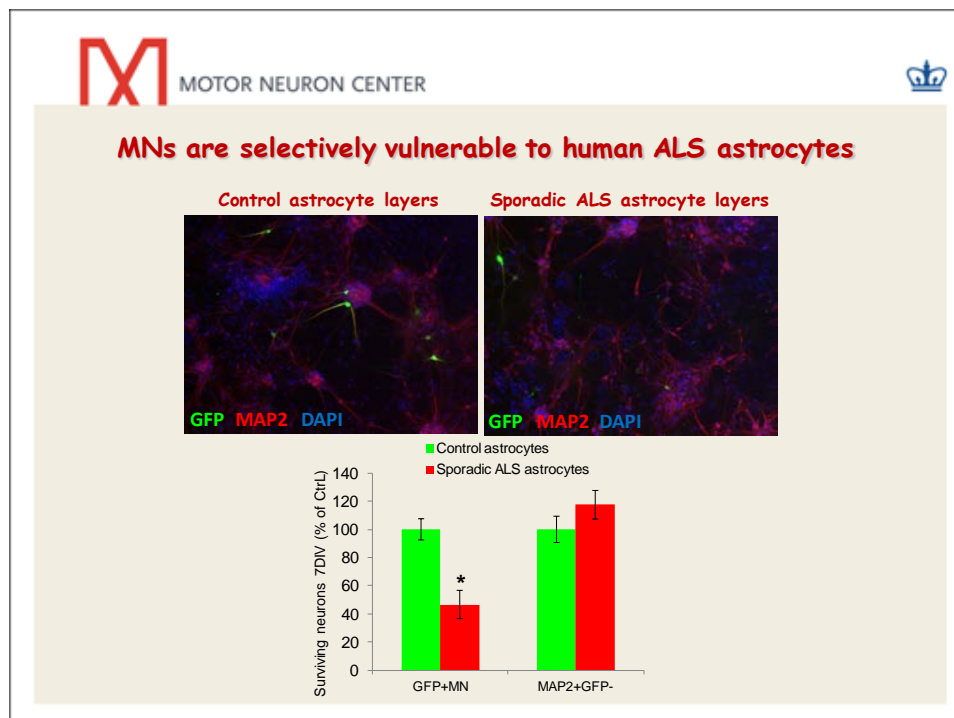


Figure 27. As demonstrated with rodent cultures, human MNs are selectively vulnerable to human mutant SOD1 astrocytes.

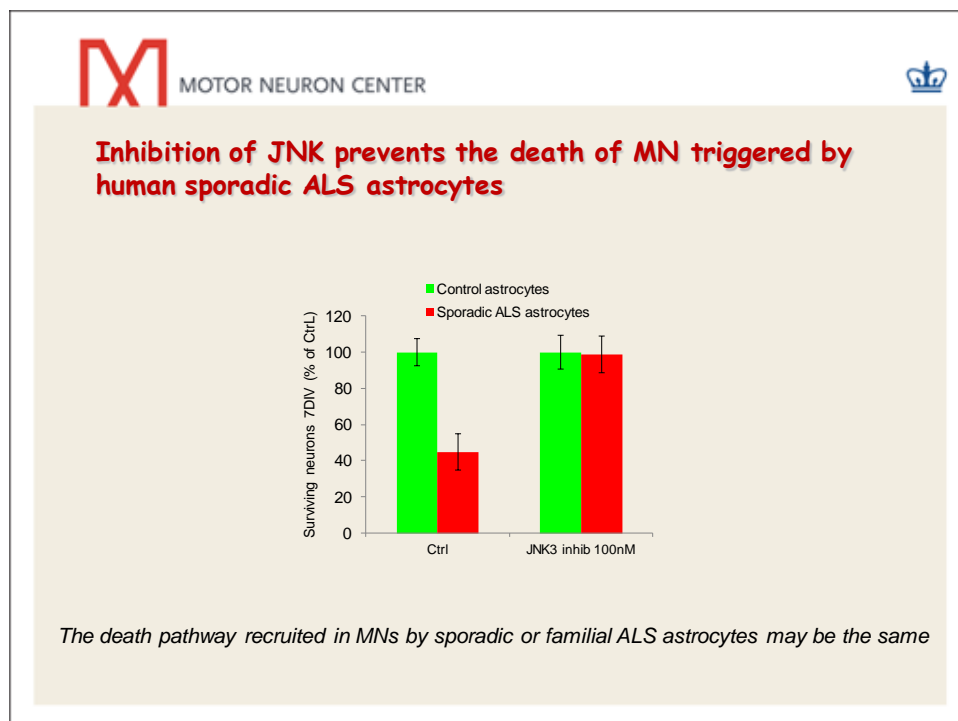


Figure 28. JNK1 inhibition in human astrocyte-MN co-cultures exhibits the same neuroprotective behavior as seen in rodent co-cultures.

Reportable Outcomes.

Year 01 (2008-2009)

Our co-culture system is validated

We can produce our SOD1 mutant astrocyte medium in large quantities.

SOD1 mutant astrocyte-conditioned medium is toxic to both MNs and ES-MNs.

We can now purify ES-MNs by fluorescence-activated cell sorting (FACS).

Mutant astrocyte-derived toxicity is enhanced by ion exchange purification.

The Flash Cytometer used to assess MN growth and survival.

Working on steps to miniaturization of our co-culture model to a 96-well plate assay.

Year 02 (2009-2010)

Verified and replicated the co-culture assay system

Miniaturized the co-culture assay system to a 96 well plate

Z'-factor is Improved from 0.8 to 0.23.

Second screen: Low throughput screen confirmed that JNK2/3 inhibitors rescued primary motor neurons whereas ERK, p38 and MLK inhibitors did not.

Second screen: Bax inhibition through the use of V5 is protective to the motor neurons. Only primary motor neurons from Bax knockout mice resistant to mutant SOD1 toxicity; primary motor neurons from Bim and Bak knockout are not.

Inhibition of p53 is not protective to motor neurons

Necrostatin-1 shown to be protective in the high throughput screen

Low throughput screen confirmed Necrostatin as protective to motor neurons in a dose-dependent manner.

Year 03 (2010-2011)

1. Primary screen

Further improvement in the quality of the assay Z'-factor from 0.23 to +0.30 by replacement of plate coating and cell density;

Improved cost-efficiency by using cortical astrocytes, new culture medium M199 instead of Neurobasal, and new benchmark compound, JNK1 inhibitor instead of Necrostatin;

Began testing of a more targeted library of biologically active compounds.

2. Secondary Screen

Low throughput screen confirmed that JNK2/3 inhibitors rescued primary MNs whereas ERK, p38, nNos and caspase 8 inhibitors did not;

Bax inhibition, through the use of V5, is protective to the MNs. Only primary MNs from Bax knockout mice were resistant to mutant SOD1 toxicity; primary MNs from Bim, Bak, p53 knockout were not;

Low throughput showed that the small molecule necrostatin-1 is protective to MNs in a dose-dependent manner.

Year 04 (2011-2012)

A total of 108 hits from the targeted library

Successful culturing of human spinal cord astrocytes and of human spinal motor neurons.

ACM from human astrocytes shows toxicity to spinal motor neurons similar to that seen in our animal studies with ALS mice.

JNK inhibition seems to protect human MNs against astrocyte toxicity in human ALS.

Discussion

This project on ALS stems from our findings that rodent astrocytes expressing mutated SOD1 kill spinal primary and embryonic mouse stem cell-derived motor neurons (27) and that this mutant astrocyte-induced spinal motor neuron death seems to be triggered by a soluble toxic factor(s) through a Bax-dependent mechanism (27). However, SOD1 mutant astrocytes do not cause the death of spinal GABAergic or dorsal root ganglion neurons or of embryonic stem cell-derived interneurons. In contrast to SOD1 mutant astrocytes, fibroblasts, microglia, cortical neurons and myocytes expressing mutated SOD1 do not cause overt neurotoxicity (27). These findings indicate that astrocytes may play a role in the specific degeneration of spinal motor neurons in ALS. While we are trying to identify the toxic factor(s), we are also trying to identify small molecules that may help to alleviate the symptoms of ALS and maybe ALS itself. To do this, we have developed an ES-MN/SOD1 mutant astrocyte co-culture system which we believe can be useful for the screening of small molecules. Essentially, we use medium that has been conditioned with SOD1 mutant astrocytes. MNs placed in this medium will die in about a week. Interestingly, MN death is, at the most, about 50%. The screening method which we are setting up will simply contain ES-MNs, mutant SOD1 astrocyte-conditioned medium (ACM) and a reasonable concentration of a small molecule to be tested. This system is faster than using animals because it is really a preclinical screen as this assay gives a possibility score, which allows the compound to go to the next level. There were four levels to this project, which involved the development of our high throughput screening technique. The beauty of this high throughput screen is that it is far less time-consuming and much more cost-effective than using living animals. And, in today's environment, where it takes about eight years to bring a drug to the market, this type of assay for drug possibilities is a necessity.

In setting up this co-culture high throughput screening technique (*Level 1*), our first task was to validate our co-culture system. To this end, we have validated our co-culture system so that we know that the components of the assay work well. The astrocyte-conditioned medium that we are using is toxic to both MNs and ES-MNs. Since we can now make a significant number of ES-MNs, far greater than the number of MNs that can be cultured from mouse spinal cord MNs, and we can make the ACM in large quantities, this co-culture system represents advancement in the way that drugs will be tested and at the speed at which they are tested. These are important for there is an urgency to do something to ease even the symptoms of ALS and we were to screen two chemical libraries containing several thousand chemicals. Purifying the ES-MNs using fluorescence-activated cell sorting (FACS) by removing unneeded and unwanted substances makes the system better in that removal of unnecessary substances except for the ES-MNs enhances the system and probably lowers background readings. Furthermore, our explanation of our miniaturization process in detail allows one to understand the process of the development of our high throughput assay. Moreover,

while our particular high throughput drug screen is specific for ALS, it is not inconceivable that our system can be adapted for drug screens for other diseases.

Validation of our new system required us to not only redo our original work but to also extend our studies to exclude other cell types by demonstrating that these cells did not produce a toxic factor nor were they susceptible to any ACM. We replicated the fact that only the mutant astrocyte-conditioned medium was toxic to primary spinal motor neurons and to embryonic stem cell-derived motor neurons (ES-MN) as well. Furthermore, in time-response studies, during 14 days of co-culture with mutant SOD1 astrocytes, MNs exhibit a selective vulnerability compared to other neurons such as Lim2-positive interneurons and other MAP2-positive GFP-negative neurons. Thus, we can conclude with certainty that MNs are selectively vulnerable to the astrocyte conditioned medium. Interestingly, regardless of whether the cells being exposed are MNs or ES-MNs, the toxic effect is the same. The toxic effect is the same even when the conditioned medium is renewed. This demonstrates that we can use ES-MNs rather than spinal MNs as making these ES-MNs represents an endless supply of MNs, even though they are stem cell derived thus, the readily expandable nature of the system.

Level 2 of this project is concerned with replication and reliability which are extremely important issues when developing any assay. Most important about this year's work is that the high throughput screen was miniaturized and that we increased the Z'-factor of our assay system. To guide us in the optimization of our assay, we followed the value of the Z'-factor (32) which best reflects the quality of the assay under normal distribution (33). We subjected our data for the positive and negative controls, which are used to calculate the Z'-factor, to a Kolmogorov-Smirnov normality test. Thus far, neither the data for the positive nor for the negative controls diverge significantly ($p > 0.05$) from a normal distribution. In our first set of experiments for this part of the work, we obtained a Z'-factor of -0.06, which is a sign of a poor quality assay. An assay with a Z'-factor of 0.0-0.5 is considered a marginal assay and an assay with a Z'-factor of 0.5-1.0 tells us that the assay is good. In analyzing our first and, I must say, very disappointing results, we found that one of the main reasons for this poor Z'-factor was the narrowness of the dynamic range and the low S/B (signal to background noise) ratio of our current assay. Indeed, the difference between our positive and negative control (ES-MN numbers in wild-type and mutant conditioned medium, respectively) was only slightly greater than the value of the negative control itself (192 vs. 186 cells) which gave an S/B ratio of ~2. We have recently found that processing mutant astrocyte-conditioned media through a Q Sepharose™ XL strong anion exchange was capturing the toxic activity, thus enabling us to concentrate the ACM to increase MN toxicity at 7 DIV. With this chromatographic step, the S/B ratio jumped from ~2 to ~3 which is at least where we wish to be with the S/B ratio to obtain a high quality assay that operates at an excellent statistical power of 0.80. Now, when we recalculated the Z'-factor with our current CV of 15% and S/B ratio of ~3, we obtained a Z'-factor of 0.23 which was a huge improvement in our assay, but it was still below our desired (and recommended) target of 0.50 as discussed above. Right now, our Z'-factor has been increased to 0.3. This is due to our replacement of the plate coating that we were using and an increase in cell density per well in the plates. Under our last set of values, we can also calculate the power

Minimization of the co-culture assay. Yes, we still have to get to the 0.5 or more level for the Z'-factor and we are still working and will continue to work on improving this number.

We work on more than one front at the same time for our high throughput assay. In the past, we have used our co-culture system to look into what pathways might be involved in the death pathways of MNs in ALS. On a *3rd level*, we have identified JNK2/3 inhibitors as protective to MNs. JNK seems to have a role in apoptosis, which points to the death of the large motor neurons in ALS as possibly being apoptotic. On the high throughput screen, Bax inhibition via V5 was noted to be protective. In addition, when MNs from Bax, Bim and Bak knockout mice were used with mutant ACM, only Bax knockout primary MNs were not affected by mutant SOD1 toxicity. Usually Bax and p53 work together (35), however in this case, inhibition of p53 was ineffective in protecting MNs. Thus, it was surmised that Bax activation here must be via another pathway, i.e. p73 activation by JNK (36). We also found with the high throughput screen that Necrostatin-1 offered protection. Determining the levels of Necrostatin-1 in the blood and the tissues has been a bit of a challenge, however we are working to get this problem resolved. But, if JNK inhibition proves to be a better avenue than Necrostatin, we will most likely go this route.

Accompanying the high throughput screen is the low throughput screen. This screen is about the toxicokinetics and pharmacokinetics of the compounds being put through the high throughput screen. Through the low throughput screen, information is garnered about the toxicokinetics and the pharmacokinetics of the compound being subjected to the high throughput screen. It is performed in conjunction with the high throughput screen. A secondary low throughput screen confirmed that JNK2/3 inhibitors are effective for protection to MNs and it has already been shown that JNK inhibition reduces neuronal death and increases behavioral outcome in neonatal brain injury (37). Our high throughput screen of the referenced small molecule library has thus far netted 108 hits which will be tested further to gauge their suitability for further investigation as a possible treatment for ALS.

These last years, we have successfully produced primary astrocytes made from two sporadic ALS and four control (non-neurological diseases) patients. With these cultures, we evidenced that human sporadic ALS and control astrocytes exhibit the classical astrocyte markers (glial fibrillary acidic protein and A2B5), the astrocyte morphology of classical ALS and controls, and an index of viability. Human sporadic ALS astrocytes are toxic to co-cultured mouse motor neurons in contrast to astrocytes produced from control patients. Furthermore, like the astrocytes from mutant ALS mice, human ALS astrocyte toxicity is selective for motor neurons as other neurons in the dish were not affected. Moreover, we noted that human astrocyte-conditioned medium was toxic to cultured human motor neurons. Our preliminary data also suggest that the death pathway that is recruited in motor neurons exposed to rodent astrocytes is the same for human ALS motor neurons exposed to mutant human astrocytes as inhibitors of JNKs in the human death pathways are equally protective in both models. We are currently trying to determine whether the observed toxicity underlies the release of the toxic soluble factors. Altogether, work here suggests that our finding of a role for astrocytes in

motor neuron death in ALS rodents may be relevant to the human disease condition and, more important, common to the familial and the sporadic forms of ALS. Besides, this is absolutely important if we wish to replace the rodent astrocyte-MN co-culture system with a human astrocyte-MN co-culture system for the high throughput testing of compounds that may be helpful in treating ALS, once we work out all of the kinks.

Conclusion

Normally, it takes eight years or so to get an effective drug on the market (ref) and, even then, the drug may have some problems and can still be pulled off of the market. Pre-clinical studies in animals are necessary however, the toxicokinetic and pharmacokinetic studies, that are performed in animals now, can be done in a more efficient way. Ideas as to how this can be achieved are the subjects of much debate and this project which was a four year developmental project of a system for the fast screening of drug libraries. The entire high throughput idea was put together because we had a real interest in speeding up needed drug testing of compounds to treat ALS. Other high throughput systems have been set up to screen drugs that might be beneficial in ALS. For instance, Somalinga et al (38) uses a β -Gal-based protein solubility assay which follows fluorescence. And, Benmohamed et al (39) developed a cellular model in which the readout is the blocking of mutant SOD1-induced aggregation. Furthermore, Wright et al (40) used the inhibition of SOD1 as their readout. What these methods lack is simplicity. As we have indicated above, we have already identified 108 hits as possibles for the treatment of ALS using our development-in-progress co-culture high through screening method to screen large drug libraries of already existing compounds. The screen components of our high throughput screening method are simple: astrocytes and spinal motor neurons. Our screening process seems to work reasonably well even though the Z'-factor is still not optimal. Screening of existing compounds makes so much more sense as a number of these compounds already have FDA approval or have existing toxico-pharmacokinetic screens. The only need with many of these compounds is to examine whether they can protect ES-MNs against the damaging effects of mutant ACM. And, the co-culture system that we have developed and are now working out the kinks can do this. We think that four years of work on this project has left us closer to the reality of a fast drug screening process for ALS that can probably be modified to screen drugs for other neurodegenerative diseases for which there is also no cure.

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